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13. ABSTRACT *(Maximum 200*

The implications for determining whether *wnt*-5a and *wnt*-4 act as mediators of normal cell growth are relevant to the detection, diagnosis, and the treatment of breast cancer. That is, it is important ultimately to understand whether the inappropriate downregulation of certain *wnt*-genes that are spatially-temporally expressed in developing mammary glands, such as *wnt*-5a and *wnt*-4, leads to loss of growth control. C57MG mammary epithelial cells have recently been found to endogenously express *wnt*-5a and *wnt*-4 whose RNA levels are reduced in the presence *wnt*-gene family members which transform this cell line. The intent of this proposal has been to develop new cell lines by transfecting C57MG cells with DNA *wnt*-5a and/or *wnt*-4 constructs which express antisense or sense RNA to determinethe effect on cell transformation will occur. Results found in experiments outlined in this proposal are consistent with the importance for normal *wnt*-5a and/or wnt-4 RNA transcripts levels in directing C57MG mammary epithelial cell phenotype, including cell proliferation and transformation. **This has important ramifications for breast cancer since it is known that *wnt*-5a and *wnt*-4 genes are downregulated in human breast cancer tissue and in human breast malignant cell lines.**

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Daniel [illegible] 9/15/98

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Table of Contents

Introduction

Wnt-gene family

The sixteen known members of the *wnt* family of genes express growth factor-like molecules which are thought to be implicated in mammary tumorigenesis and in early developmental events. The *wnt*-1 gene was first identified in mouse mammary tumor virus (MMTV) induced tumors occurring often in certain mouse strains (1). Analysis of these tumors revealed that MMTV proviral integration occurred in close proximity to either the 5' or 3' region of the *wnt*-1 gene locus, presumably activating *wnt*-1 through MMTV enhancer sequences (2). Since these insertions do not interrupt the protein coding sequences (3), there is expression of an intact protein. Therefore, inappropriate expression of the normal *wnt*-1 protein is implicated in cellular transformation. *Wnt*-2 and *Wnt*-3 have also been isolated from several mouse mammary tumors with activated MMTV provirus (4).

Wnt-gene mechanism of action

The mechanism of action of *wnt* genes is unknown, although several characteristics have been reported which provide insight into how their products may be important. Analysis of genomic (3) and cDNA (5) sequences has revealed that mouse *wnt*-1 encodes a cysteine rich 370 amino acid polypeptide with a leader sequence. The leader sequence, which has a potential peptidase cleavage site after amino acid 27 (6), appears to be necessary for protein function (7). Previous studies suggest that *wnt*-1 protein enters the secretory pathway, is glycosylated, and may be secreted (8), but remains strongly bound to the cell surface and/or extracellular matix (9,10), possibly to heparin (10), and is seldom found in the medium of cultured cells (9). This suggests that *wnt*-1 acts locally, possibly in an autocrine or paracrine manner. This is strengthened by the finding that mouse *wnt*-1 when transfected into 3T3 fibroblasts, which results in no obvious phenotype, can yet transform the mammary epithelial cell-line C57MG when co-cultured (11). This may be related to competence modification of cells by *wnt* signals to growth factors, including bFGF and activin (12). It has been speculated that this may occur at least in part through *wnt*-mediated gap junctional communication (13-15) as a result of influencing the expression of β-catenin to ultimately enhance $Ca2^{+}$ dependent cadherin-associated cell adhesion (16).

Wnt-genes in mammary cell differentiation and in tumorigenesis

Besides the ability of the expression of certain *wnt*s to determine cell competence during pattern formation in vertebrate development, an *in vivo* role for MMTV directed *wnt*-1 expression in mammary tumorigenesis has been directly shown in transgenic mice (17). More recently, *wnt*-3 has also been found to be transcriptionally activated by MMTV provirus in mouse mammary carcinomas (7). In addition, *wnt*-2 has been implicated in mammary tumorigenesis since it is overexpressed and amplified in transplanted virally induced tumors (4). In cell culture systems, the ectopic expression of *wnt*-1 or *wnt*-2 is able to transform certain mammary epithelial cell lines (7), including C57MGs derived from normal mammary gland of a C57 B1/6 mouse (18). However, little is known how various members of the *wnt*-gene are capable of transforming mammary epithelial cells and are involved in mammary tumorigenesis. Some investigators believe that *wnt*-1 and other transforming *wnt*-gene peptides act on endogenous *wnt*-gene signalling pathways to transform cell types.

Recent findings by Gavin and McMahon (19) have demonstrated that at least five

members of the sixteen known mouse *wnt*-genes, including *wnt*-5a and *wnt*-4, are differentially expressed during the postnatal development of the mouse mammary gland implicating their importance in mammary development. That is, *wnt*-5a and *wnt*-4 and other *wnt*s are expressed in virgin glands and during early to mid-pregnancy. As pregnancy approaches term, these same genes become nonexpressive and other *wnt*-genes become concomittantly transcriptionally activated (19, 20). **This interplay between various *wnt*-gene family members in mammary gland development may have implications for how *wnt*-1 and other *wnt* genes are able to transform cells. The type of *wnt* gene ultimately expressed may dictate the competence of the cell to respond to environmental factors and thus cell growth.**

C57 MG cells have been found to endogenously express *wnt*-5a and *wnt*-4 (21), and the reduction of endogenous expression of these two genes correlates with cell transformation (21). Loss of *wnt*-5a and/or *wnt*-4 transcriptional expression occurs in the presence of *wnt*-1, *wnt*-2, hepatocyte growth factor, bFGF and neu-T (c-erbB-2) (21, 22). **This suggests that *wnt*-5a and *wnt*-4 may play an important role in normal cell growth and that loss of normal gene expression may be important in cell proliferation and transformation.**

Wnt-genes in human cancer

Various members of the *wnt*-gene family have also been implicated in human tumorigenesis. *Wnt*-2, *wnt*-4, and *wnt*-7b gene expression is aberrantly increased in benign and malignant breast tumors and cell lines (23). Human *wnt*-5a in several malignant cell lines is also minimally expressed or aberrantly expressed compared to corresponding normal cell lines (24,25). Although *wnt*-5a gene expression in breast cancer tissue is somewhat elevated, its expression is markedly reduced when compared to corresponding benign tissue (25). That *wnt*-5a expression is elevated in benign tumors including colon adenomas suggests that *wnt*-5a may be functionally related to the early progression of tumorigenesis (24). It is also possible that *wnt*-5a regulates normal cell growth and its expression will transiently increase in transformed cells which is reflecting an attempt to re-establish normalcy. It is of interest that human *wnt*-5a in several normal and malignant cell lines aberrantly express without gene amplification or rearrangement compared to corresponding normal cell lines (24). It has been suggested that the upregulation of *wnt*-5a gene expression in malignant and benign tissue relates to an attempt to regulate cell migration, and that *wnt*-5a downregulation corresponds with increased cell motility (22) due to loss of cell adhesion (26-28).

The short arm of chromosome 3 (3p) in human cancer

Genetic alterations of chromosomes containing tumor supressor genes are thought to be contributing to the multi-stage progression of malignant tumors (29). These alterations may include nonrandom chromosomal deletions or loss of heterozygosity (LOH) (30). The short arm of chromosome 3 (3p) has a particularly high frequency of deletion or rearrangement in human cancers including small cell lung carcinoma, oral squamous cell carcinoma, cervical carcinoma, breast carcinoma, renal cell carcinoma, and uroepithelial cell carcinoma (31-36).

In human renal cell carcinoma, loss of alleles and cytogenetic aberrations have been well documented. For example, up to 89% of nonpapillary forms of renal cell carcinomas have been shown to have a nonrandom loss of chromosome 3p (37,38). The loss of

heterozygosity determined by restriction fragment length polymorphism (RFLP) analysis occurs consistently in renal cell carcinoma suggesting the loss of one or more tumor suppressor genes which likely play a significant role in renal cell carcinogenesis (36). Although the precise location of the 3p tumor suppressor gene(s) is not known, cytogenetic analysis suggests that the region 3p11-3p25 likely carries one or more suppressor genes (36,39, 40). More specifically, Yamakawa et al. mapped one suppressor gene to 3p13-p14.2 and another distal to 3p21.3 in renal cell carcinoma (41). Another region encompassing 3p12-p14 has been found to dramatically alter tumor growth in nude mice when a fragment containing this region was introduced into a highly malignant nonpapillary renal cell carcinoma cell line (42). This region includes the translocation breakpoint in familial renal cell carcinoma (35,43,44). A nontumorigenic human renal cell carcinoma cell line (RCC23) has been established from a stage III nonpapillary carcinoma with a loss of heterozygosity (LOH) on 3p (45). Chromosome and RFLP analysis revealed an unbalanced translocation between chromosome 3p and 8q (t(3;8)(p11;q11)) resulting in the loss of the 3p11-pter region (45). Using RCC23 cells, previous studies showed that microcell hybrids containing an introduced intact chromosome 3 resulted in a significant reduction in growth rate, saturation density, and altered morphologic phenotype compared to the parental cancer cells (45). Furthermore, it was later demonstrated that the introduction of chromosome 3 also restored cellular senescence which was associated with repression of telomerase activity (46). Importantly, most of these findings have been mimicked in RCC23 cells ectopically expressing *wnt*-5a as discussed in Section C, Preliminary Studies.

In bladder cancers, a specific correlation between the loss of chromosome 3p and the development of high grade malignancy has recently been found (35). In support of the hypothesis that genes on chromosome 3p act as tumor suppressors, Wu et al. showed that all somatic cell hybrids which were formed between nontumorigenic SV-HUC-1 cells and an isogeneic derivative transitional cell carcinoma cell line (MC-T16) that lost 3p on initial transformation were tumorigenically suppressed (47,48). Upon reversion, hybrids were subsequently found to have a deletion of chromosome 3p13-p21.2 (49).

Human *wnt*-5a has been cloned and mapped to human chromosome 3p14-3p21 (24,50). Preliminary studies reported below suggests that *wnt*-5a is a growth regulating gene and possibly a candidate tumor suppressor gene which may be deleted or rearranged during the multi-step development of events which lead to tumorigenesis. **The experiments outlined in this proposal have important implications for understanding basic mechanisms underlying tumorigenesis and indicate that the deregulated expression of a *wnt*-5a may be important in the multi-step progression of cancer.**

Differential display of eukarotic mRNA by PCR

Normal cellular development and abnormal growth patterns occur as a result of changes in gene expression. Determining altered patterns of gene expression is critical to uncovering underlying mechanisms of action important to normal growth control. Methods are now available to distinguish patterns of gene expression between two comparable sets of mRNAs include those that are based on subtractive hybridization. This technique, however, has several disadvantages in that it is cumbersome and requires a great deal of time and personnel committment. An alternative technique known as differential display (51) has several technical advantages for screening genes that are differentially regulated. It is quicker, allows simultaneous detection of two or more groups of differentially expressed genes, and is PCR-based allowing for the use of small amount of RNA. **The differential display method allows the investigator to visualize mRNA by displaying subsets of parallel mRNA from different but comparable origins as short cDNA bands.** These cDNAs can then be quickly sequenced and compared directly to databanks, and can also be amplified by PCR and

used as probes for Northern or Southern blotting or to isolate genes from cDNA or genomic libraries. Applications of RNA fingerprinting to tumors have included the identification of differentially expressed genes between normal and tumor cells (52). The advantage of this technique becomes apparent when a cloned gene found by arbitrarily primed differentially display from specific RNA becomes identified through database analysis. Any information about its known function in other systems offers directions in determining regulatory mechanisms with which the gene is involved.

In this proposal, it is planned to compare by differential display newly constructed cell lines with and without *wnt*-5a as a single variable. If *wnt*-5a is a growth regulating gene, and indeed is a candidate tumor suppressor gene, comparing differential gene expression should provide for a systematic approach for uncovering novel mechanisms of action for *wnt*-5a signalling pathways.

Significance

The proposed research is significant in its application of modern molecular techniques to answer fundamental questions about the function of *wnt*-5a in cell growth and differentiation. This becomes particularly relevant to human cancer if it can be demonstrated that the loss, mutation, or rearrangement of *wnt*-5a in malignant cell lines occurs and can be reverted by *wnt*-5a gene replacement. This grant proposal presents a series of experiments which will determine the role of *wnt*-5a in the multi-step progression of tumorigenesis. Preliminary data already generated (discussed below) strongly supports an important role for *wnt*-5a in cell transformation and in tumorigenesis. In summary, this proposal to test the specific hypothesis that *wnt*-5a is a growth regulating gene with tumor suppressor function will likely provide for expanding our understanding of *wnt*-gene function in human cancers. This is directly relevant to the pursuit of information on the causal basis for how transformed cells develop and expand into malignant tumors.

Body of report

Progress on Specific Aim 1: Determine whether anti-sense wnt-5a in C57MG mouse mammary epithelial cells leads to cell transformation and tumorigenesis.

The mammalian expression vector pRSV was blunt ligated to the mouse *wnt*-5a cDNA full length clone (gift of A. McMahon, Harvard) in the antisense orientation which was confirmed by restriction enzyme mapping. The above mammalian expression vector was co-transfected with pSV2NEO (gift of J. Papkoff, Sugen, Palo Alto, Ca) into C57MG mammary epithelial cells using lipofection as described previously (53). Stable cell lines were selected and then maintained in 250 ug/ml G-418 in Dulbecco's modified media supplemented with 5% defined bovine serum and 5% fetal bovine serum. Several clones expressing antisense *wnt*-5a constitutively in C57MG cells were screened and confirmed by Northern blot analysis using mouse *wnt*-5a sense riboprobes to hybridize 20 ug of isolated total RNA from confluent cell cultures. The effect of antisense *wnt*-5a ectopically expression on endogenous *wnt*-5a and RNA levels were done as shown in Figure 1.

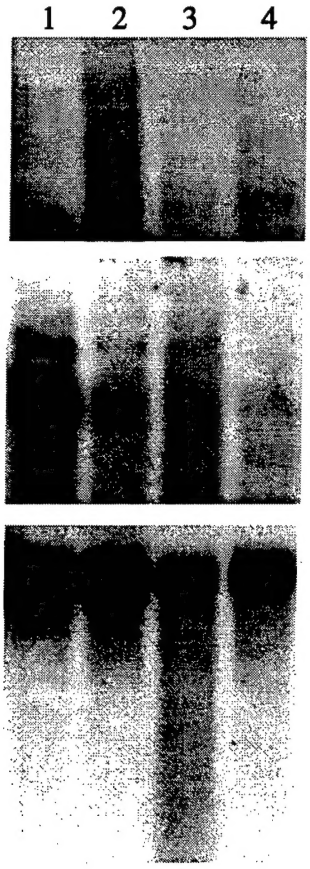


Fig 1. Northern blot probed with sense *wnt*-5a riboprobe for antisense *wnt*-5a expression in top panel compared to the same blot probed with antisense *wnt*-5a riboprobe to determine endogenous *wnt*-5a in middle panel. Lower panel is the same blot probed with actin for RNA loading. Lane 1 is control C57MG cells while lanes 2-4 are three different clones of C57MG cells expressing antisense *wnt*-5a.

A morphology assay was then done by plating 10^5 cells/plate of parental C57MGs, *wnt*-1 expressing C57MGs (known to downregulate *wnt*-5a), and C57MGs expressing antisense*wnt*-5a and a photograph taken at confluence as shown in Figure 2 below.

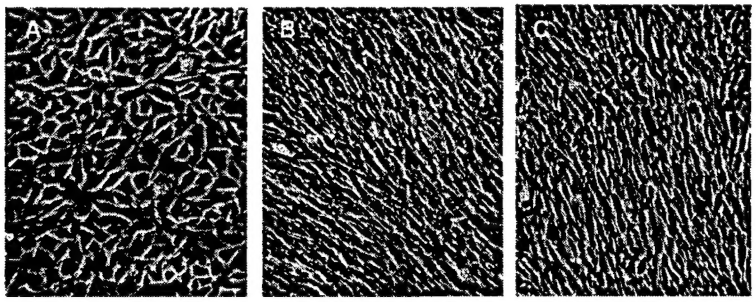


Fig. 2. Photomicrographs of C57MG cells (A) at confluence compared to C57MG cells expressing *wnt*-1 (B) and C57MG cells expressing anti-sense *wnt*-5a (C).

Clones expressing antisense *wnt*-5a were remarkably similar to the transformed cell phenotype mediated by the ectopic expression of *wnt*-1 (which downregulates endogenous *wnt*-5a in this cell line). Growth curves were done by plating 4 x 10^4 cells and cell counts taken every day for nine days (Figure 3). C57MG cells expressing antisense *wnt*-5a had growth characteristics similar to that found in *wnt*-1 transformed cells (11). ^{3}H-thymidine incorporation was consistent with the presence of antisense *wnt*-5a allowing for loss of normal cell growth and differentiation (data not shown).

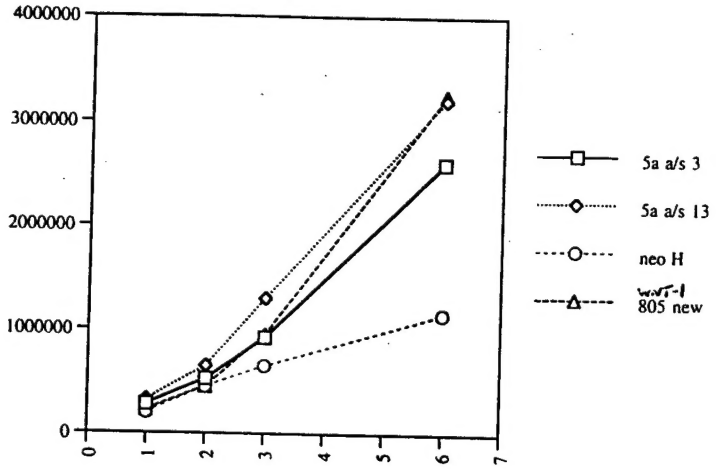


Fig. 3. Growth curves comparing C57MG parental cells with C57MG cells expressing *wnt*-1 and with C57MG cells expressing antisense *wnt*-5a.

In summary, the preliminary findings in Specific Aim #1 are **consistent with the hypopthesis that *wnt*-5a in C57MG cells is important to normal cell growth.** The loss of endogenous *wnt*-5a gene expression in the presence of antisense *wnt*-5a leads to cell transformation in a manner similar to the overexpression of *wnt*-1, or *wnt*-2 in this cell line.

Progress on Specific Aim #2: Transfection of RCC23 renal carcinoma cells with wnt-5a.

Full length human *wnt*-5a cDNA (clone T11) (50) was the kind gift of Dr. Renato Iozzo (Jefferson Medical College, Philadelphia, PA). The cDNA was subcloned into pRSV (Dr. Jackie Papkoff, Sugen, Redwood City, CA) and orientation determined by restriction analysis. RCC23 renal cell carcinoma cells characterized previously (45) were cultured in RPMI media supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin. Cells were grown to 80% confluence before each passage. For gene transfection, passage 20 cells were grown to 50% confluence and the media exchanged for low serum media (Optimem, Gibco). Using 90 µl liposomes (Lipofectin, Gibco) mixed with 10 µg of pRSV*wnt*-5a and/or pSV2neo in a total volume of 150 µl, the cells were transfected overnight at 37^{o}C in 5% CO_2. The media was replaced and the cells grown overnight in media with 1% FBS without the addition of Geneticin (G-418, Gibco). The cells were then selected in media supplemented with 800 ug/ml G-418. Individual clones were isolated, resistant colonies expanded into cell lines, and maintained

in media supplemented with 250 µg/ml G-418 for eventual RNA extraction to determine gene expression of *wnt*-5a.

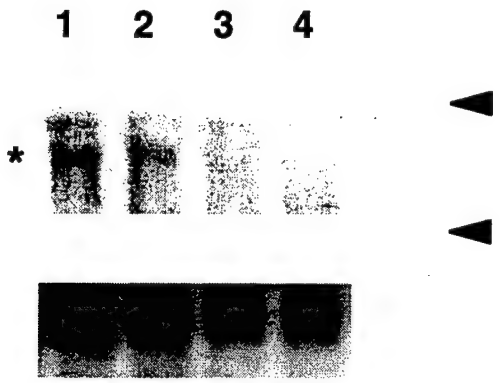


Fig. 4. RNA blot comparing RCC23 renal carcinoma cells (lane 4) to 3 different clones of RCC23 cells expressing ectopic *wnt*-5a (lanes 1-3). The blot was reprobed for actin for RNA loading and integrity.

Morphology was determined by allowing the cells to grow to confluence and photographed as shown in Figure 5. The cells ectopically expressing *wnt*-5a have a more differentiated cell phenotype. Significantly, the changes in cell morphology mimicked that found when RCC23 cells (which are missing chromosome 3p) were utilized for microcell fusions with chromosome 3p.

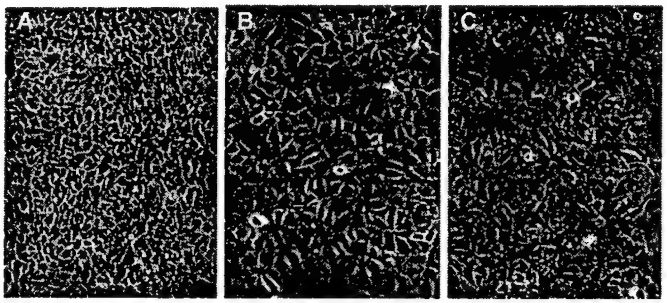


Fig. 5. Photomicrographs of RCC23 renal carcinoma cells which are missing chromosome 3p (A) at confluence compared to two clones of RCC23 cells ectopically expressing *wnt*-5a (B and C).

To determine the cell saturation density, population doubling time, and morphologic phenotype of RCC23/neo cells and RCC23/*wnt*-5a cells, cells were plated in 12-well dishes at a density of 4×10^4 cells per well. The cells were counted every day for 10 days, and the growth rate and population doubling time determined from the logarithmic part of the growth curve. The saturation density was determined from the cell number after the cells reached confluence at 10 days. These experiments were repeated three times with similar results.

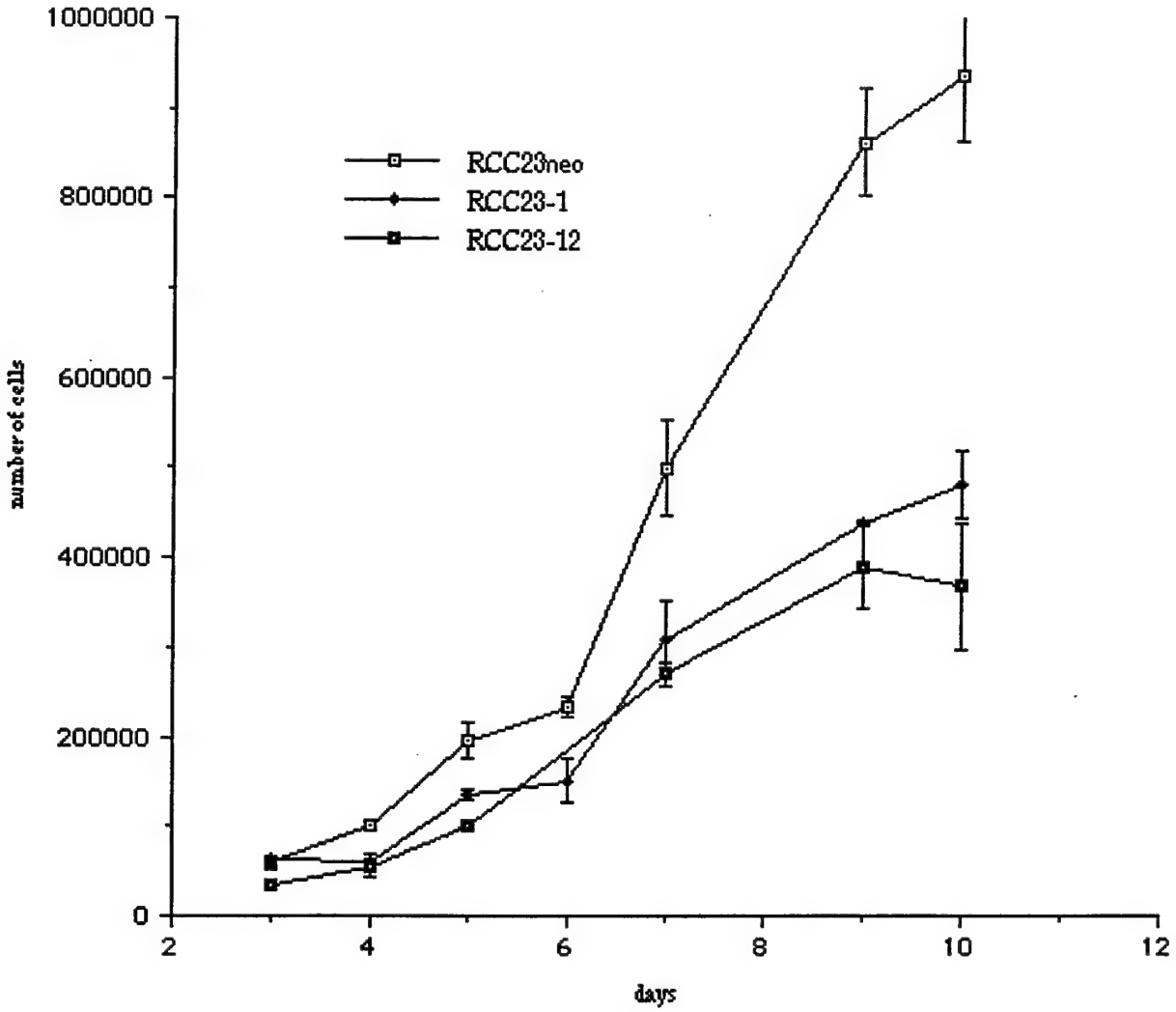


Fig. 6. Growth curves of RCC23 cells compared to two different clones of RCC23 cell ectopically expressing *wnt*-5a.

Transfection of MC-T16 uroepithelial cancer cells with *wnt*-5a.

Uroepithelial carcinoma cells (MC-T16) were transfected with full length human *wnt*-5a using liposomes (Lipofectin, Gibco, BRL). Clones were stably selected in G-418, isolated, and expanded into stable cell lines. Northern analysis using a random primed *wnt*-5a cDNA ^{32}P-labeled probe demonstrated expression of the expected 3.2 kb size RNA in several clonally expanded cell lines. Two clones expressing *wnt*-5a (Fig. 1) were selected for comparison to the MC-T16/neo transformed and SV-HUC-1/neo nontumorigenic immortal cell lines resistant to G-418.

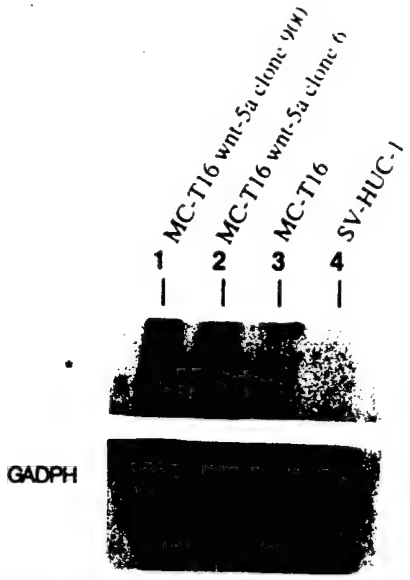


Fig. 7. RNA blot comparing MC-T16 uroepithelial cancer cells expressing ectopic *wnt*-5a (lane 1, clone 900 and lane 2, clone 6) to MC-T16 cells (lane 3) and to parental SV-HUC-1 (lane 4).

The growth properties were compared between the MC-T16/neo parental cells and two clones (Fig. 7, lanes 6 and 8) of MC-T16/*wnt*-5a to determine whether *wnt*-5a had any influence on growth kinetics. As shown in (Table 1) and (Fig. 8), the growth rate of the MC-T16/neo cells expressing *wnt*-5a was similar to MC-T16 cells in the logarithmic phase unlike that for SV-HUC-1/neo cells and unlike that found in RCC23/*wnt*-5a cells (Fig. 6). That is, the population doubling time of MC-T16/neo cells was 26 hours while that for MC-T16/*wnt*-5a-clone 6 cells was 32 hours and 24 hours for MC-T16/*wnt*-5a-clone 900, in comparison to the doubling time for SV-HUC-1/neo cells which was 56 hours. Cell saturation density at confluence correlated with growth rate. That is, MC-T16/neo parental cells had a saturation density of 8.9×10^5 at confluence, while both MC-T16 clones expressing *wnt*-5a had saturation densities of 9.4×10^5 and 8.0×10^5. This was significantly different than that observed for SV-HUC-1/neo cells which had a saturation density of 3.2×10^5. **Unlike that found for RCC23/*wnt*-5a cell lines, comparing these results suggests that the expression of *wnt*-5a in MC-T16 cells does not alter growth kinetics significantly. This may be due to the immortalization of these cells by SV-40 which is not altered in the presence of *wnt*-5a.**

Table 1 *Growth characteristics of MC-T16 cells transfected with human wnt-5a.*

Cell type	Generation time (h)	Saturation density (total cells x 10^5)	^{3}H-thymidine incorporation (cpms)	Colony formation (%)
SV-HUC-1	56	3.2 +/- 0.1	8,212	0
MC-T16	26	8.9 +/- 0.8	15,110	9.7 +/- 0.3
MC-T16 hw*nt*-5a-6	32	9.4 +/- 0.7	11,420	0
MC-T16 hw*nt*-5a-900	24	8.0 +/- 0.6	9,435	0.06 +/- 0.03

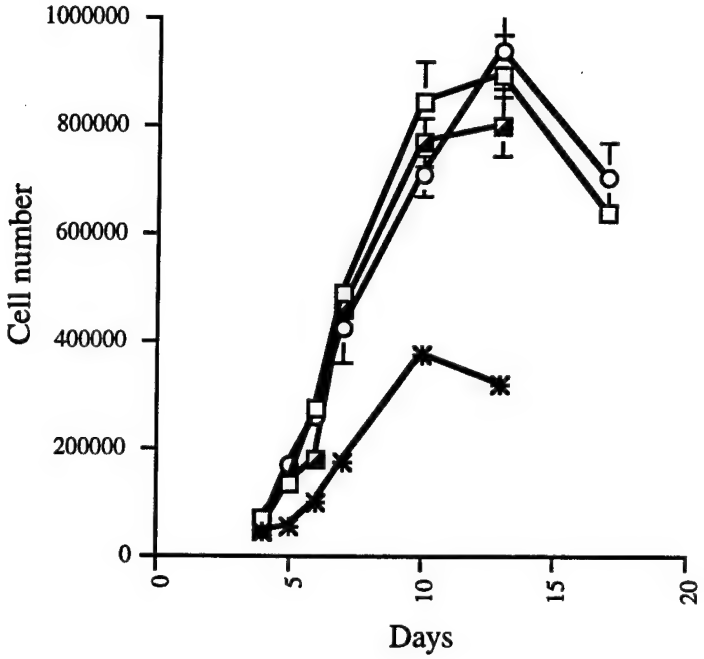


Fig. 8. Growth curves of MC-T16 uroepithelial cancer cells compared to MC-T16 cells ectopically expressing *wnt*-5a and to parental SV-HUC-1 cells (*).

Morphologic differences in MC-T16/*wnt*-5a cells. The parental SV-HUC-1/neo cells characteristically retain many of the features associated with normal epithelial cells in culture. The isolated SV-HUC-1/neo clone used for these experiments no longer grew at confluence (Fig. 9b) unlike that for SV-HUC-1/neo pooled clones which become tightly packed at confluence (unpublished observations). MC-T16/neo carcinoma cells continue to grow at confluence, are refractive, and have a spindle cell phenotype (Fig. 9a). However, when two different clones of MC-T16/*wnt*-5a cells were examined, the cells at confluence become less spindle shaped, more flattened, and pleiomorphic (Fig. 9c and d) similar to the SV-HUC-1/neo cells. **These findings suggest that MC-T16 bladder cancer cells ectopically expressing *wnt*-5a are more differentiated than the MC-T16/neo cells.**

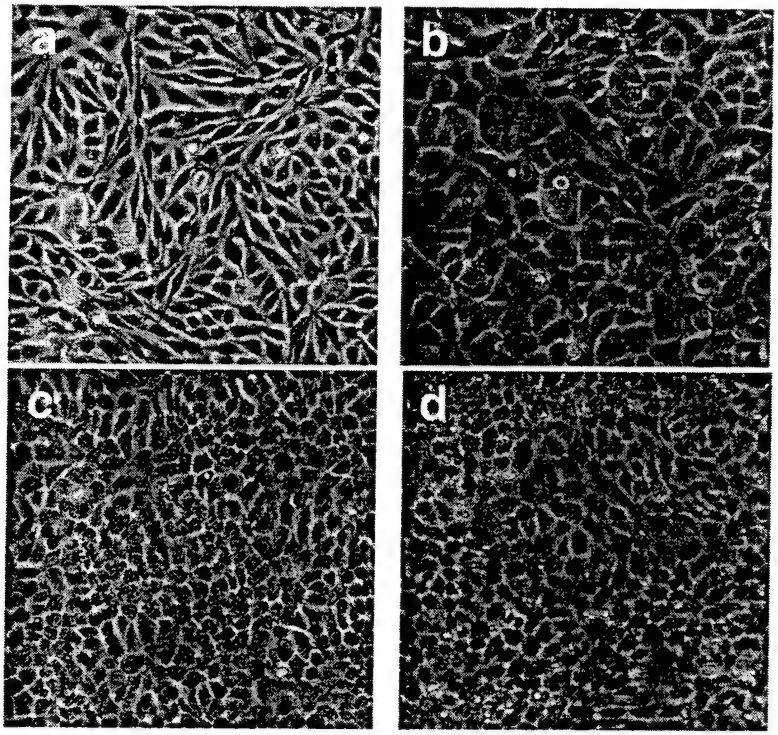


Fig. 9. Photomicrographs of MC-T16 uroepithelial cancer cells (a) compared to parental SV-HUC-1 (b) and to two clones of MC-T16 cells ectopically expressing *wnt*-5a (c, clone 6 and d, clone 900).

Anchorage dependance assay. MC-T16/neo cells grow in 0.35% agar with a cloning efficiency of 9.7% compared to no growth in corresponding parental G-418 selected SV-HUC-1/neo cells (Table 2, below). The expression of *wnt*-5a in MC-T16 cells re-establishes contact dependent growth under these conditions in two different clones. The experiments were repeated three times with similar results.

Table 2. *Soft agar assay comparing cloning efficiencies*

T16 malignant cells	T16-*wnt*5a clone 6	T16-*wnt*5a clone 900	SV-HUC-1 normal cells
9.6%	0%	0.06%	0%

Tumorigenesis in athymic nude mice. Subcutaneous inoculation of MC-T16/neo uroepithelial cells resulted in tumor formation after 4 weeks in 9/10 animals. Spontaneous tumor regression occurred in 4 mice which has been previously described for tumors derived from this cell line (47), and one 1 cm x 1 cm tumor was removed for analysis before the end of the experiment. The remaining tumors grew slowly until the third month when tumor growth rapidly accelerated (Fig. 10). These tumors were removed when greater than 1.5 cm x 1.5 cm or became necrotic. No tumors grew in 10 mice inoculated with SV-HUC-1/neo cells or in 10 mice inoculated with MC-T16/*wnt*-5a-clone 6 cells. However, 5/5 mice inoculated with MC-T16/*wnt*5a-clone 900 cells grew tumor after a lag time of six weeks. Tumor regression occurred in one animal and one 0.6 cm x 0.8 cm necrotic tumor was removed from another animal. At three months, these tumors grew to a maximum size which never enlarged greater than 1 cm x 1 cm over the following three months (Table 3). The tumors that were examined from mice inoculated with MC-T16/neo

cells were attached to underlying tissue and were vascular, while the tumors that grew in mice inoculated with MC-T16/*wnt*-5a-clone 900 cells were found to be unattached to surrounding tissues and remarkably avascular. Tumors from both groups examined histologically confirmed the gross findings. Furthermore, the tumors expressing *wnt*-5a were 80-90% centrally necrotic (Fig. 11a) compared to MC-T16/neo cell derived tumors which were 10-20% necrotic even when considering that the latter tumors grew to a much larger size (Fig. 11b). At higher magnification, little stroma was apparent in MC-T16/*wnt*-5a (Fig. 11c) derived tumors compared to MC-T16/neo tumors (Fig. 11d).

Table 3. *Tumorigenicity of human uroepithelial cells in athymic nude*
Cells at passage 18-20 were grown and 2 x 10^6 cells in 0.1 ml were injected s.c into the right dorsal quadrant of 4-6 week old female athymic nude mice.

Cell lines	Tumor formation	Tumor regression	Tumor size > 1 cm x 1cm
SV-HUC-1	0/10	0/0	0/0
MC-T16	9/10	4/9	4/5
MC-T16 h*wnt*-5a-6	0/10	0/0	0/0
MC-T16 h*wnt*-5a-900	5/5	1/5	0/4

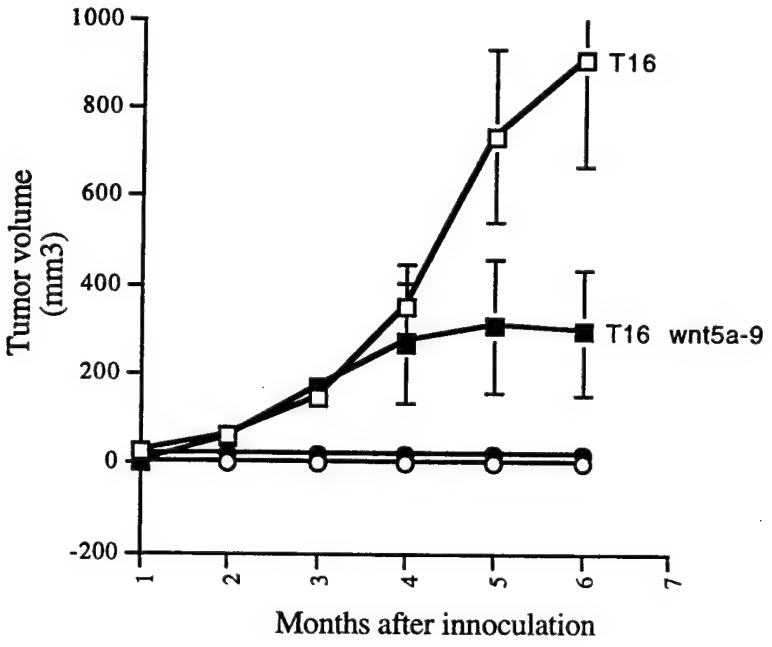


Fig. 10. Tumor volume after inoculation of MC-T16 cells into athymic nude mice compared to MC-T16 cells ectopically expressing *wnt*-5a and to parental SV-HUC-1 cells. MC-T16/wnt-5a, clone 6 and parental SV-HUC-1 tumors did not grow while MC-T16/wnt-5a clone 900 stopped growing after three months.

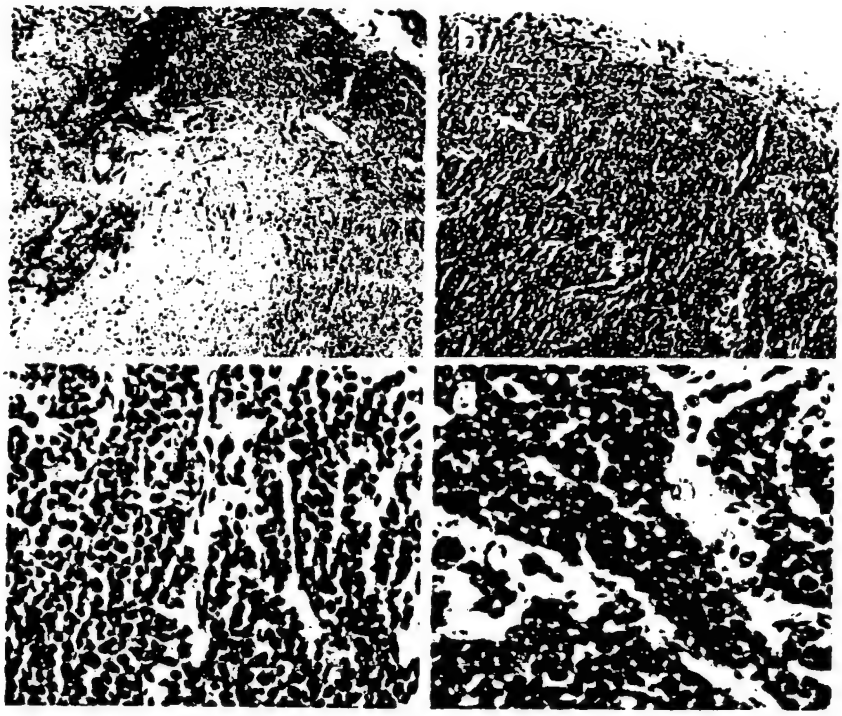


Fig. 11. Photomicrographs of (a) MC-T16/*wnt*-5a derived tumors showing extensive necrosis are compared to (b) MC-T16 uroepithelial cell derived tumors with no necrosis. Higher magnification of MC-T16/*wnt*-5a cells (c) illustrates the lack of stroma when compared to the same magnification of MC-T16 tumors (d) with obvious stroma.

Telomerase assay. Reports have shown that the process of transformation/neoplasia is associated with the activation of telomerase, a ribonucleoprotein enzyme complex that adds telomeric repeats (hexanucleotide 5'-TTAGGG-3') to the ends of replicating chromosomes, or telomeres (53–56), and that another member of the *Wnt* family (57) has been shown to regulate telomerase. We compared telomerase activity between two different G-418 resistant clones of parental MC-T16/neo cells and two clones of MC-T16/*wnt*-5a cells by the primer extension telomere repeat amplification protocol (TRAP) assay in which telomerase synthesizes telomeric repeats onto oligonucleotide primers (58,59). Results indicate no change in telomerase activity in either MC-T16/*wnt*-5a expressing cell lines as shown in Figure.12.

However, **telomerase activity when compared between two different clones of G-418 resistant clones of RCC23 cells to two RCC23 cell lines expressing *wnt*-5a indicate a 5-fold inhibition of telomerase activity** in both RCC23/*wnt*-5a expressing clones as shown in Figure 12, comparing lanes 3, 5, 7, and 9. This discrepancy in the repression of telomerase activity by *wnt*-5a in MC-T16 cells most

likely relates to the immortalization of MC-T16 cells by SV-40 which is known to activate teloemerase enzyme activity (79).

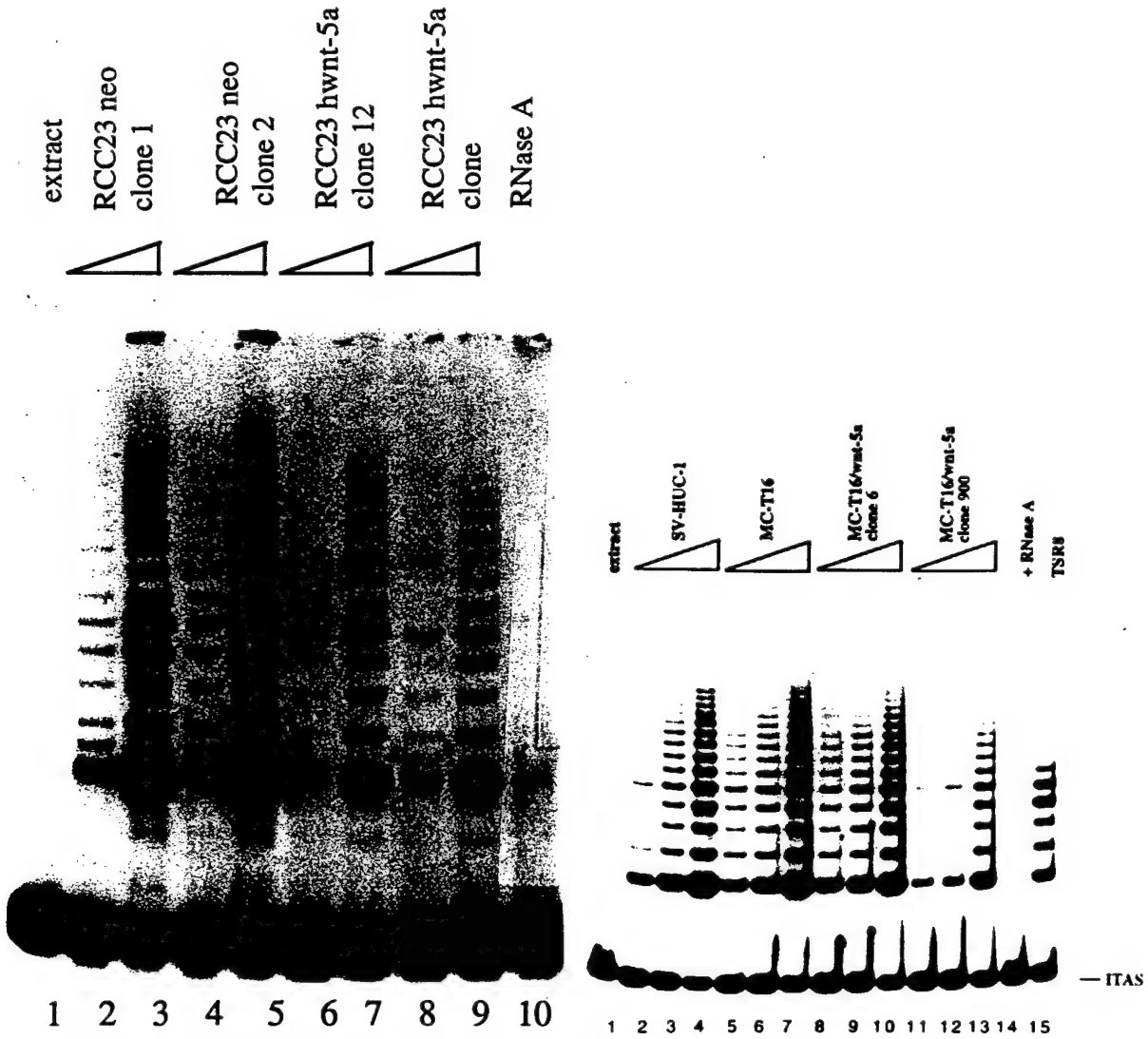


Fig. 12. Telomerase enzyme activity by the primer extension telomere repeat amplification protocol (TRAP) assay in which telomerase synthesizes telomeric repeats onto oligonucleotide primers. RCC23 renal cancer cells (A) expressing *wnt*-5a show telomerase repression compared to MC-T16 uroepithelial cancer cells expressing *wnt*-5a (B) which do not show repression.

Determining the whether *wnt*-5a affects cell motility. It has been reported that *wnt*-5a is capable of inhibiting cell motility and migration in two different biologic systems (22, 26, 27). Considering the preliminary findings on reversion of tumorigenesis after inoculation of MC-T16 cells expressing *wnt*-5a *in vivo,* it becomes important to determine in this cell line whether *wnt*-5a alters cell motility. An *in vitro* wound healing assay to ascertain *wnt*-5a effects on cell migration was done using MC-T16 cells compared to SV-HUC-1 parental cells and to MC-T16 cells expressing *wnt*-5a. After 48 hours post-wounding of confluent cell cultures, it was evident that the ectopic expression of *wnt*-5a limits cell motility across wounds as seen in Figure 13. This suggests that one direct effect of ectopic *wnt*-5a in this cell line is to compromise cell migration which ultimately could compromise tumor growth.

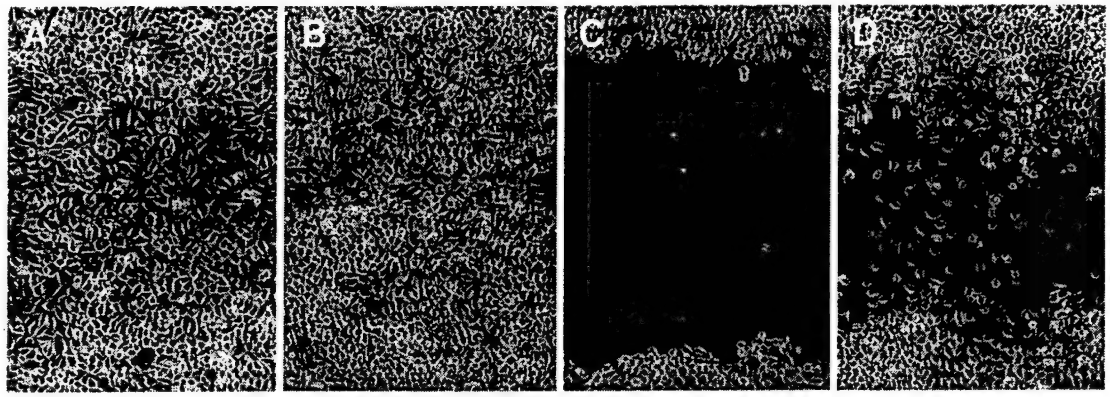
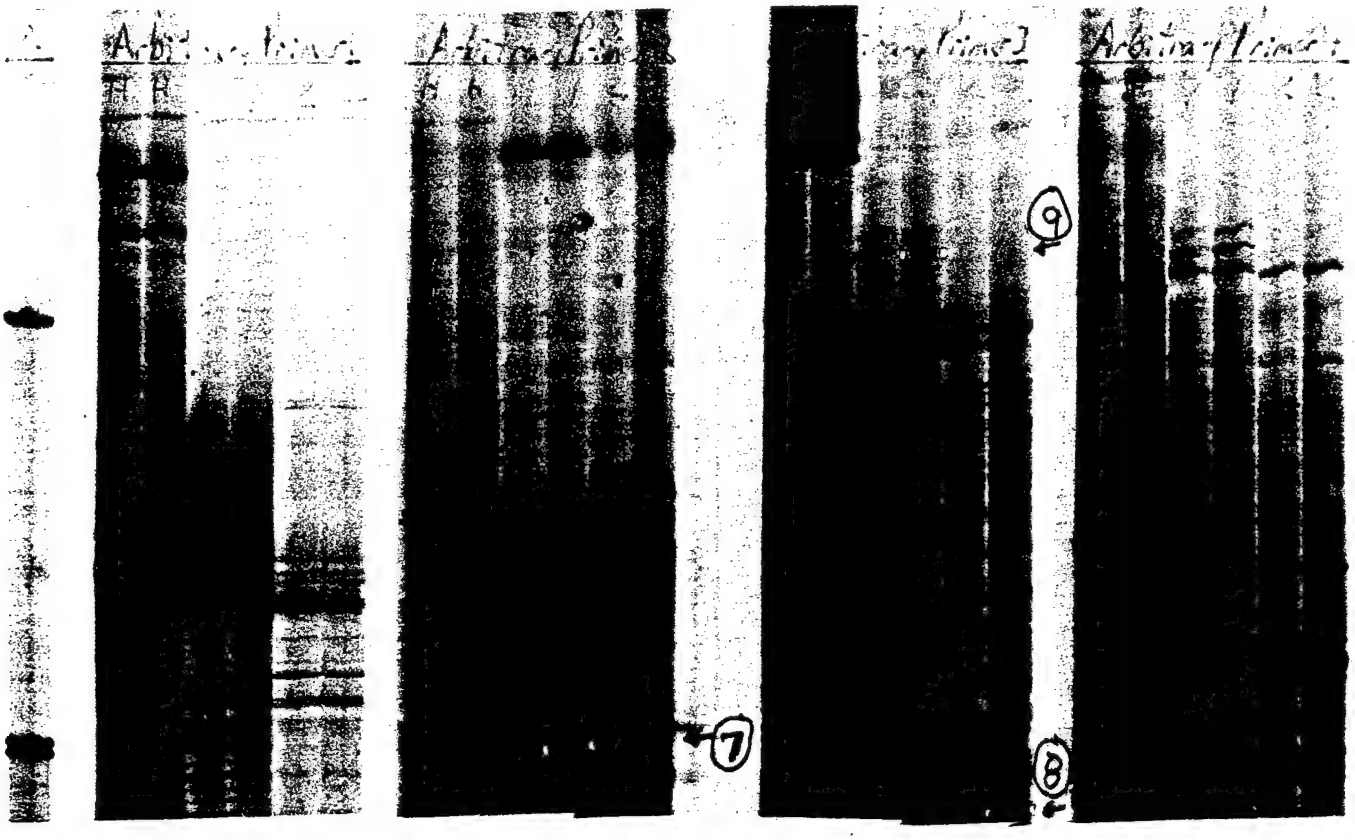


Fig. 13. Photomicrographs of a wound healing assay at 48 hours after wounding confluent cells of SV-HUC-1 (A) compared to MC-T16 uroepithelial cancer cells (B) and to two clones of MC-T16 cells ectopically expressing *wnt*-5a (C, clone 6) and (D, clone 900).

PCR based differential display was done on C57MG cells expressing *wnt*-1 compared to parental C57MG cells. Using arbitrary primers, cDNA samples were run in duplicate (lanes 2-5) against a positive control (lane 1) revealing several bands which were isolated and sequenced. Using BLAST, the sequences were compared to known DNA gene sequences. As shown in the figure below several candidate genes were found to be expressed only in *wnt*-1 transformed cells, the most interesting of which was homologous with an **intermediate filament** sequence. It is known that intermediate filaments in epithelial cells are not normally expressed except when these cell types transform. This is being investigated further the results of which will be reported at a later date.



Summary of Results and Discussion

During the first 30 months as outlined in the Statement of Work Task 1 and Task 2, results indicate that ectopically expressed *wnt*-5a in C57MG cells rescues the *wnt*-1 transformed phenotype. *Wnt*-4 does not appear to rescue the *wnt*-1 phenotype and much of the experimentation revolved around the effects of *wnt*-5a rather than with the effects of *wnt*-4. Furthermore, when antisense *wnt*-5a constructs were transfected into C57MG cells, transformation occured in a manner similar to that seen when *wnt*-1 is ectopically expressed in these cells. This did not occur when antisense *wnt*-4 was transfected. When both *wnt*-4 and *wnt*-5a were simultaneously re-expressed in *wnt*-1 transformed cells, the rescue phenotype was similar to that found when only *wnt*-5a but not *wnt*-4 was ectopically expressed. When antisense *wnt*-5a and *wnt*-4 were transfected into C57MG cells, the change in phenotype was similar to that seen when only antisense *wnt*-5a was expressed.**These findings indicate that the presence of *wnt*-5a in directing C57MG cell phenotype and proliferation may be the underlying mechanism of the manner in which *wnt*-1 or *wnt*-2 (which reduce the expression of endogenous *wnt*-5a in this cell type) allow for cell transformation.**

Once it became obvious that *wnt*-5a rather than *wnt*-4 seemed to be an important factor in cell growth control, the cell lines expressing *wnt*-5a or antisense *wnt*-5a were characterized using anchorage independent assays, cell kinetics, and thymidine incorporation. In summary, anchorage independent assays were negative for the cells lines tested. This was not unexpected considering *wnt*-1 transformation does not allow for loss of contact growth inhibition. When *wnt*-1 C57MG cells are transfected with *wnt*-5a, there is decreased cell saturation density at confluence which is similar to that for the parental cells. Antisense *wnt*-5a expression in normal C57MG cells changes saturation density in a manner similar to that seen when these cells express *wnt*-1. In addition, *wnt*-1 transformed cells when transfected with *wnt*-5a incorporate 3H thymidine similar to that seen for nontransformed parental cells. In contrast, when antisense *wnt*-5a constructs are transfected into normal C57MG cells, 3H thymidine incorporation is similar to those cells ectopically expressing *wnt*-1. **The data suggest that *wnt*-5a is a growth regulator of C57MG cells which is particularly relevant to the manner in which *wnt*-1 may transform this cell type. That is, *wnt*-1 may transform cells by disregulating *wnt*-5a gene expression. This has important ramifications for breast cancer since it is known that *wnt*-5a is downregulated in human breast tissue and in human breast malignant cell lines**. These studies have been reported in ***Experimental Cell Research***which is enclosed in the Appendix of the original copy of this final report.

In the **Statement of Work Task 3**, it was anticipated to determine the expression of *wnt*-5a and other *wnt*-genes in mouse mammary tumors and in human breast malignant cell lines and tissues. However, data has been published by other laboratories which indicate the expression and importance of various *wnt*-gene family members in human malignant cell lines and tumors and therefore Task 3 was altered. It was elected to direct our research toward uncovering genes that are expressed in *wnt*-1 transformed C57MG cells using PCR mediated differential display. *Wnt*-1 cells were directly compared to parental C57MG cells not expressing *wnt*-1. We have isolated several differentially expressed bands after a great deal trial and error. Eventually we utilized the services of a commerical company and sequenced several DNA bands. After sequencing the cDNA bands and after comparing them to known sequences in gene data banks using BLAST, the most interesting of these was homologous to an intermediate filament which is highly expressed in *wnt*-1 transformed cells, but not in the the parental cell. This may have relevance to the finding that *wnt*-5a stops cell motility observed in these studies. This work is ungoing and will be reported at a subsequent time.

In the **Statement of Work Task 4** determining the importance of *wnt*-5a became the focus of this investigation. It was elected to delineate further the importance of *wnt*-5a in human tumorigenesis. Much of this stemmed from knowing that *wnt*-5a had been mapped to chromosome 3p 14-21 and the consistent finding of the loss of heterozygosity of chromosome 3p in malignant tumors which have a poor prognosis. Initially, human breast cancer cells missing chromosome 3p 14-21 were transfected with human *wnt*-5a. We were not able to maintain these cells in vitro long

enough to characterize them. This was repeated 3 times without success. Therefore, other human cancer cell types missing chromosome 3p were used to determine whether *wnt*-5a could revert the transformed phenotype. Human bladder cancer cells missing chromsome 3p 14-21 were transfected with *wnt*-5a. Remarkably, the transformed phenotype was reverted in vitro and in vivo in athymic nude mice. This was reported in a recent issue of ***Cell Growth and Differentiation*** and is included in the Appendix in the original copy of this report. Human renal cancer cells missing chromosome 3p were also transfected with human *wnt*-5a. In this study an attempt was made to determine whether *wnt*-5a could be a candidate tumor suppressor gene important to altering the malignant phenotype. Not only did *wnt*-5a rescue the phenotype but also decreased telomerase activity in a manner similar to that found in hybrids transfected with chromosome 3p indicating that *wnt*-5a may indeed be a tumor suppressor gene important to renal cancer. These studies were reproted in **Tumor Biology** and a reprint is enclosed in the Appendix in the original report.

Conclusions

These experiments have clearly characterized *wnt*-5a as being extremely important in normal cell growth and differentiation. It is likely that the disruption normal RNA levels of *wnt*-5a alter cell behavior and may well lead to increases in cell proliferation and transformation. It has been disappointing that the breast cancer cell types used to transfect *wnt*-5a have been without success since the cells have continued to grow very slow and eventually die before they could be characterized. Therefore, much of the work done in human cell tumorigenesis has been done on cell types other than breast cancer cells, but the relevance is likely the same for breast cancer. We will continue to look for human breast cancer cell types missing chromosome 3p for *wnt*-5a transfection to determine more directly whether *wnt*-5a can rescue the malignant phenotype.

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APPENDIX

FIGURE LEGENDS

1. Northern blot analysis of C57MG cells transfected with antisense *wnt*-5a. The top panel are three clones stably expressing antisense *wnt*-5a compared to the parental cell line (lane 1) determined by probing the blot with a *wnt*-5a sense riboprobe. The middle panel is the effect of ectopic antisense *wnt*-5a on endogenous *wnt*-5a determined by probing with an antisense *wnt*-5a riboprobe. The lower panel is the middle panel blot normalized for RNA loading using an actin cDNA probe. By densitometry, endogenous *wnt*-5a decreases 6-fold in clone 1 (lanes 2), 8-fold in clone 2 (lane 3), and 12-fold in clone 3 (lane 4).

2. Northern blot analysis of C57MG cells expressing *wnt*-1 (known to downregulate endogenous *wnt*-5a) which have been transfected with sense *wnt*-5a. Shown are C57MG parental cells (lane 1) compared to transformed C57MG/*wnt*-1 cells (lane 2). Four C57MG/*wnt*-1 clones stably expressing sense *wnt*-5a are seen in lanes 3-6. The same blot is normalized for RNA loading using an actin cDNA probe. Three clones were chosen for further analysis (lanes 3, 5, 6).

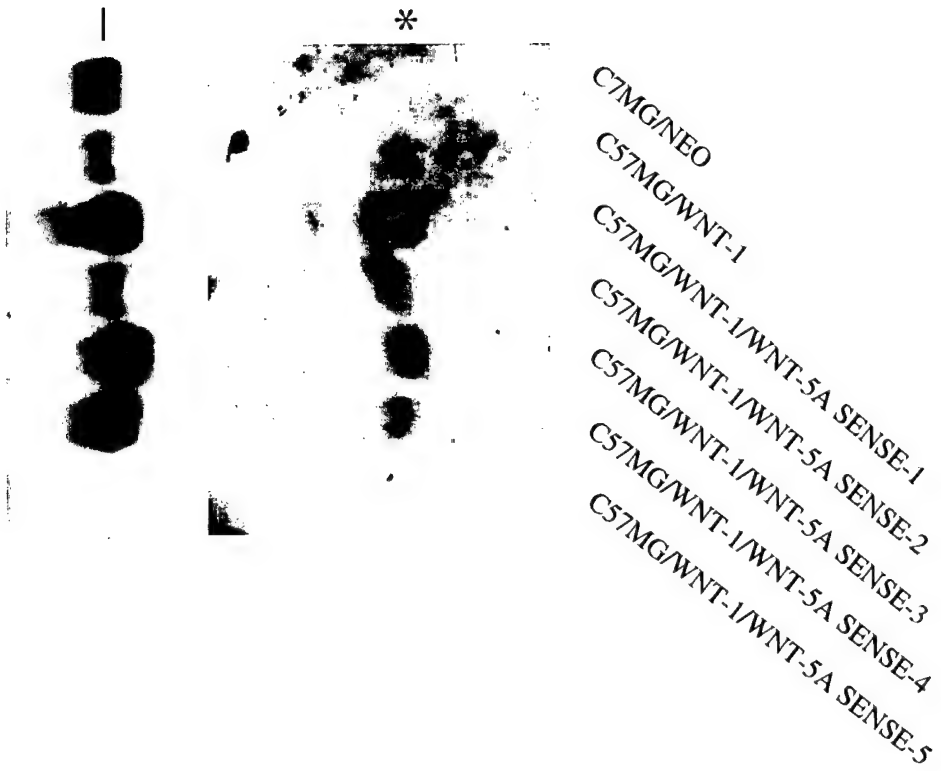
3. Photomicrographs of three clones stably expressing antisense *wnt*-5a in C57MG cells. The parental cells (A) at confluence are compared to transformed C57MG cells expressing *wnt*-1 (B) known to downregulate endogenous *wnt*-5a. Clones expressing ectopic antisense *wnt*-5a (C-F) have a morphologic phenotype similar to that seen when the cells are transformed with *wnt*-1. The highest expressing antisense *wnt*-5a clone (clone-3) (C and D) tends to form foci at confluence.

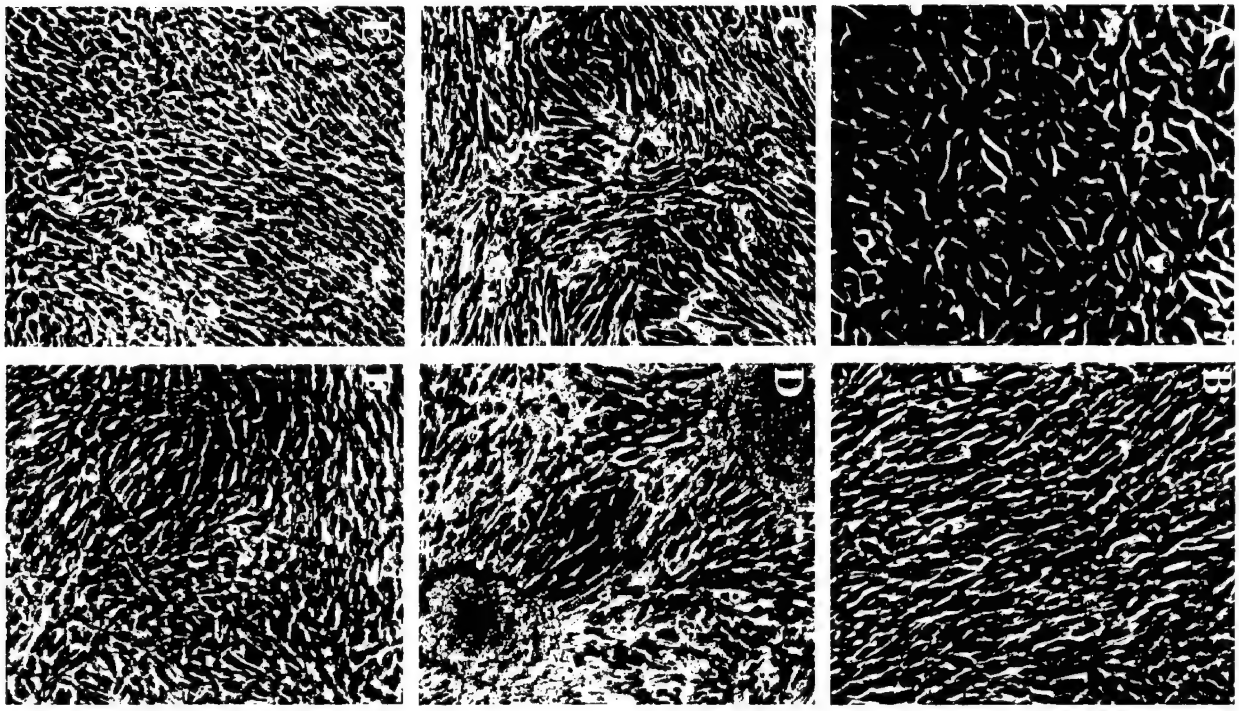
4. Photomicrographs of three clones stably expressing sense *wnt*-5a in C57MG/*wnt*-1 cells. The parental cells (B) at confluence are compared to transformed C57MG cells expressing *wnt*-1 (A) known to downregulate endogenous *wnt*-5a. Clones expressing ectopic sense *wnt*-5a (C-E) have a morphologic phenotype similar to that seen in the parental cells suggesting that ectopic *wnt*-5a can revert the *wnt*-1 transformed cell phenotype.

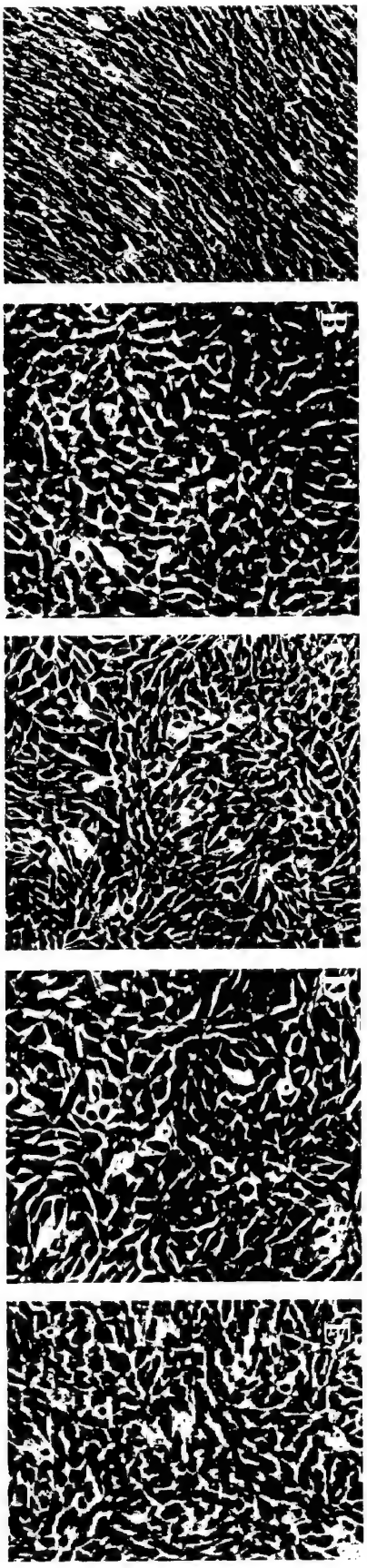
5. Growth kinetics of C57MG cells expressing antisense *wnt*-5a indicate that saturation density at confluence for all three clones is similar to that found when the cells are transfected with *wnt*-1 but unlike that for the parental cells (A). DNA synthesis in all three clones expressing antisense *wnt*-5a is increased at confluence similar to that seen when the cells are expressing *wnt*-1 but unlike that in the parental cells (B).

6. Growth kinetics of C57MG/*wnt*-1 transformed cells expressing sense *wnt*-5a indicate that the saturation density at confluence for all three clones has decreased indicating a partial reversion of cell growth when compared to parental cells (A). DNA synthesis in all three clones expressing sense *wnt*-5a is decreased at confluence similar to that seen in the parental cells (B).

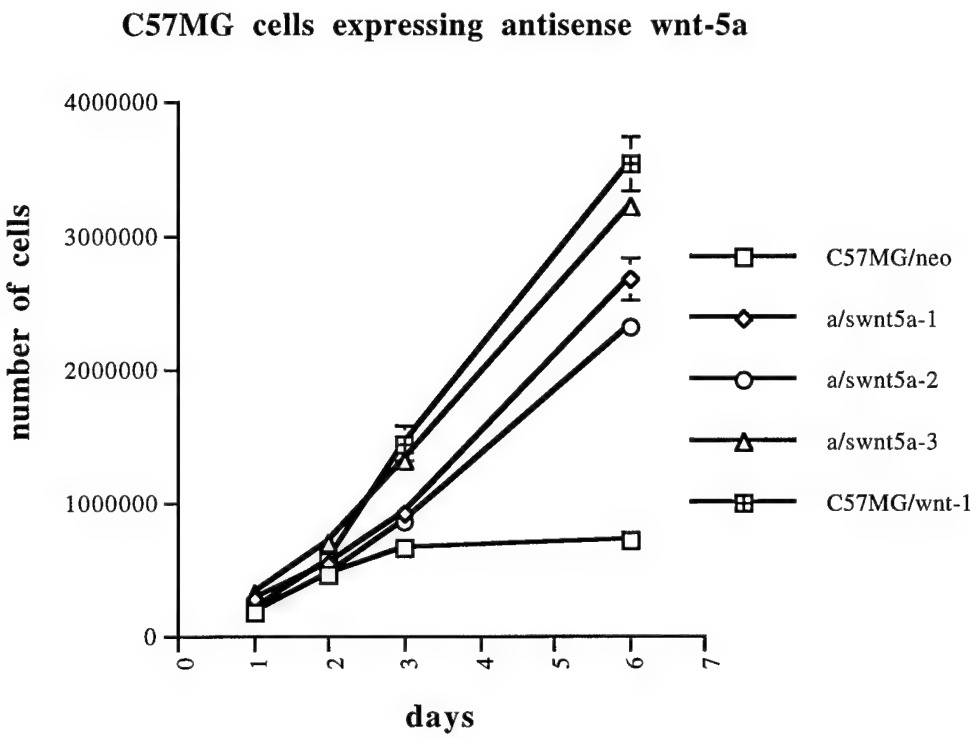

C57MG/neo
C57MGa/swnt-5a-1
C57MGa/swnt-5a-2
C57MGa/swnt-5a-3


C7MG/NEO
C57MG/WNT-1
C57MG/WNT-1/WNT-5A SENSE-1
C57MG/WNT-1/WNT-5A SENSE-2
C57MG/WNT-1/WNT-5A SENSE-3
C57MG/WNT-1/WNT-5A SENSE-4
C57MG/WNT-1/WNT-5A SENSE-5

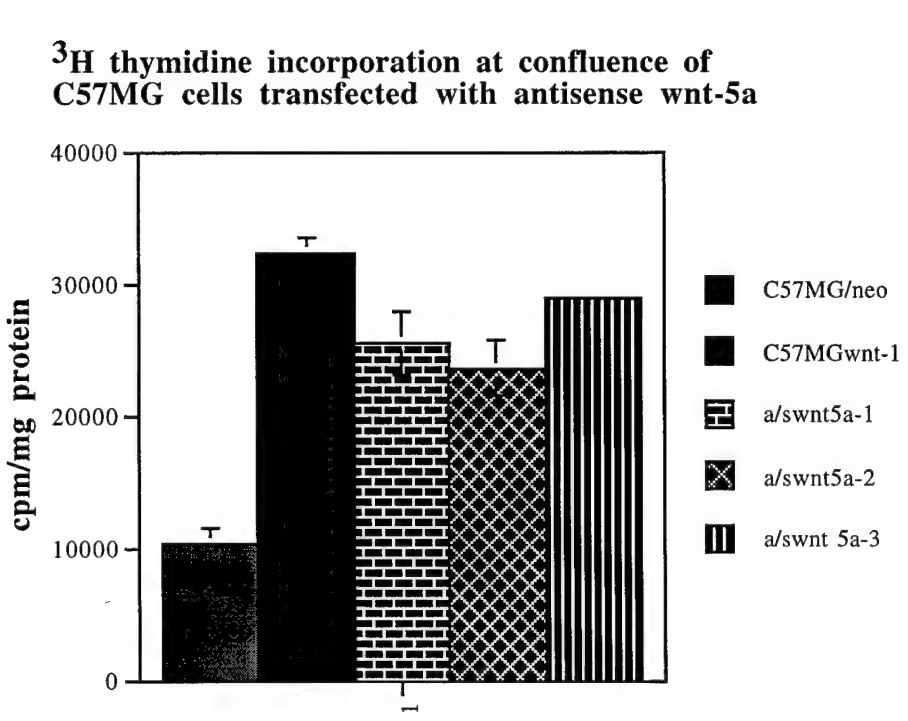

C
D
B



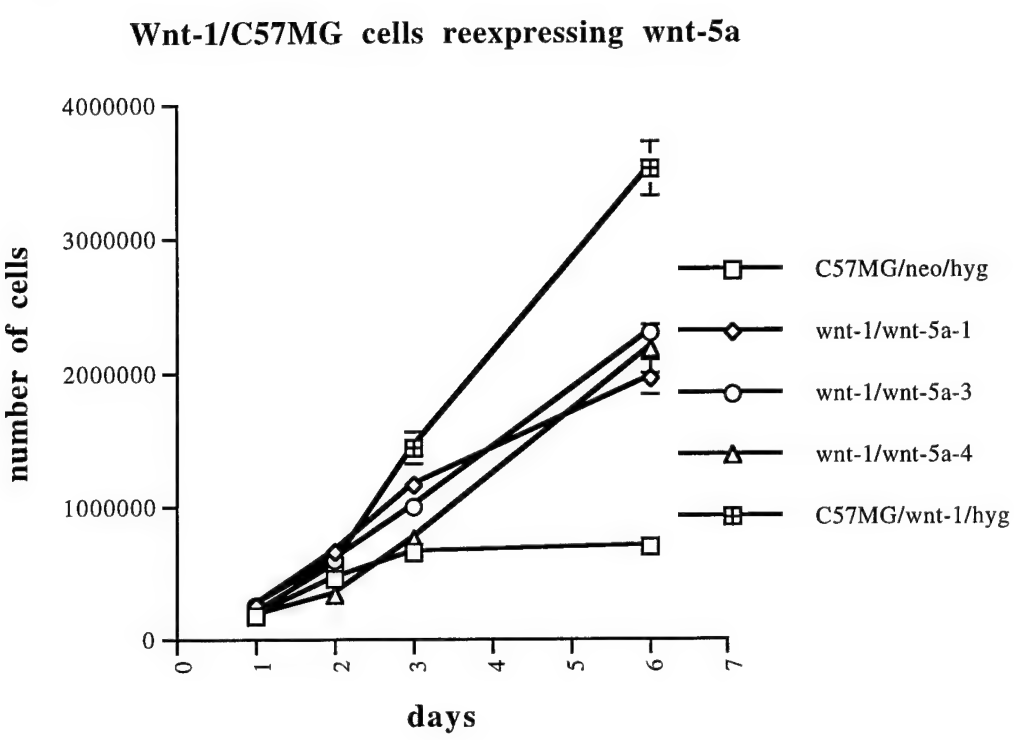
A


C57MG cells expressing antisense wnt-5a
number of cells
4000000
3000000
2000000
1000000
0
0
1
2
3
4
5
6
7
days
C57MG/neo
a/swnt5a-1
a/swnt5a-2
a/swnt5a-3
C57MG/wnt-1

B


3H thymidine incorporation at confluence of
C57MG cells transfected with antisense wnt-5a
cpm/mg protein
40000
30000
20000
10000
0
1
C57MG/neo
C57MGwnt-1
a/swnt5a-1
a/swnt5a-2
a/swnt 5a-3

A


Wnt-1/C57MG cells reexpressing wnt-5a
number of cells
4000000
3000000
2000000
1000000
0
0
1
2
3
4
5
6
7
days
C57MG/neo/hyg
wnt-1/wnt-5a-1
wnt-1/wnt-5a-3
wnt-1/wnt-5a-4
C57MG/wnt-1/hyg

B

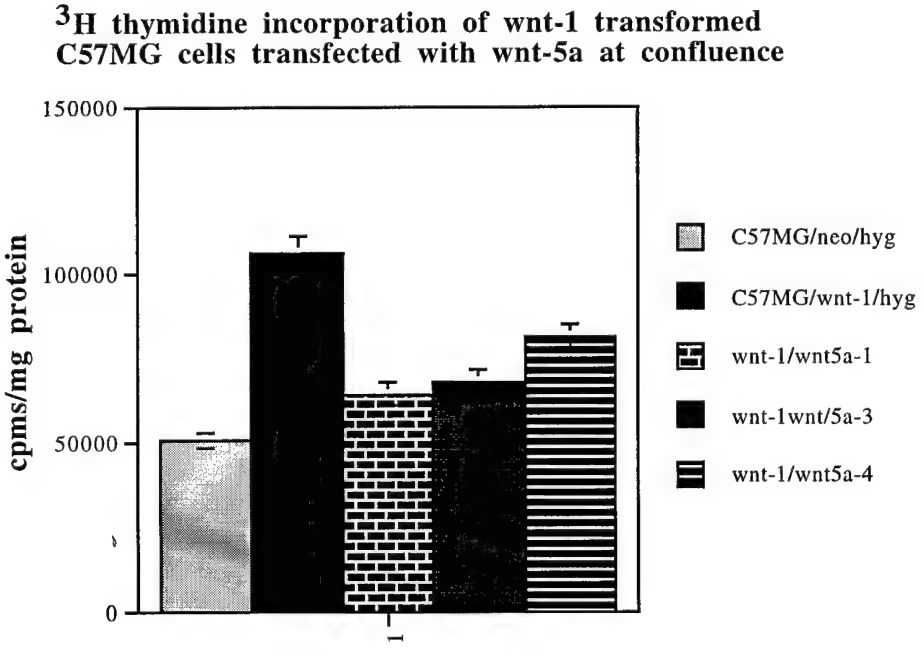

3H thymidine incorporation of wnt-1 transformed
C57MG cells transfected with wnt-5a at confluence
cpms/mg protein
150000
100000
50000
0
1
C57MG/neo/hyg
C57MG/wnt-1/hyg
wnt-1/wnt5a-1
wnt-1wnt/5a-3
wnt-1/wnt5a-4

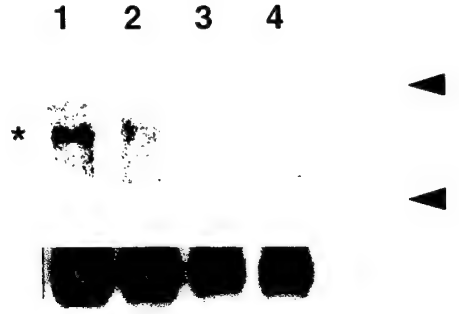
FIGURE LEGENDS

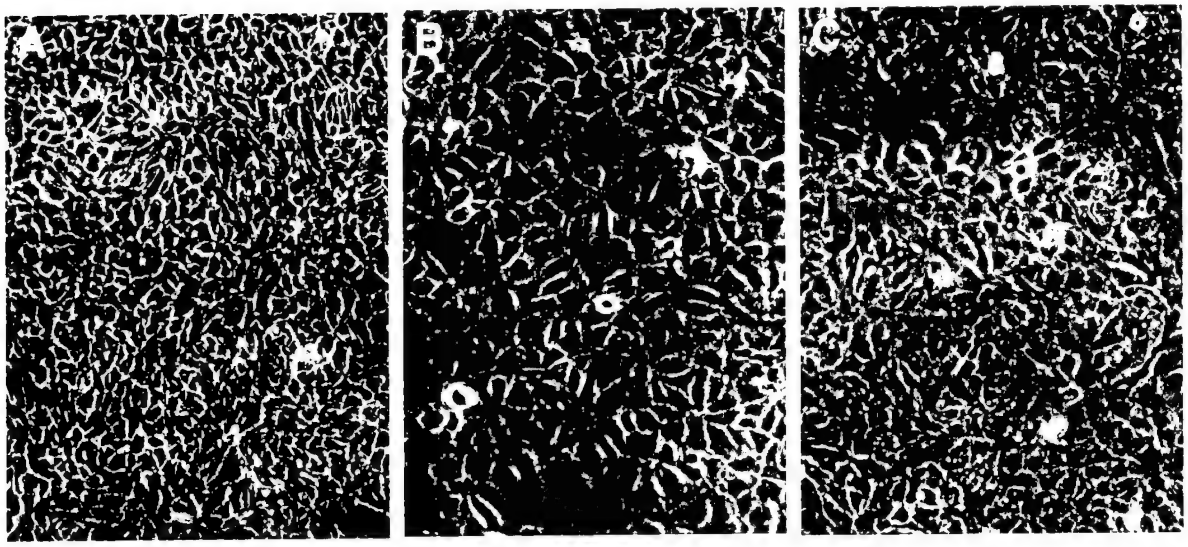
Figure 1 - Northern analysis of RCC23 cells (lane 1) and RCC23/neo cells (lane 2) compared to RCC23 cells transfected with *wnt*-5a (lanes 3-10). The same blot was probed with cyclophilin (>) for RNA loading and integrity. The asterisk (*) indicates the *wnt*-5a signal. RCC23/*wnt*-5a clone 1 (lane 4) and RCC23/*wnt*-5a clone 12 (lane 6) were selected for further analysis.

Figure 2 - Photomicrographs of confluent cells comparing two clones of stably expressing RCC23/*wnt*-5a cells to RCC23/neo cells. (A) RCC23/neo cells are refractile, tightly packed with polygonal morphology unlike RCC23/*wnt*-5a clone 1 cells (B) which are large and flat, and RCC23/*wnt*-5a clone 12 cells (C) which in addition to being large and flat have vacuolarization.

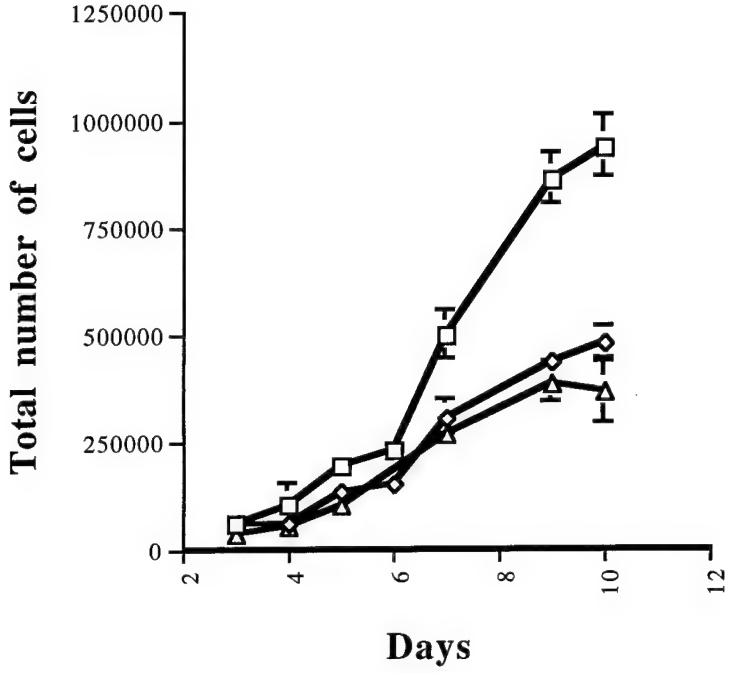
Figure 3 - Growth kinetics of RCC23 cells compared to RCC23 cells ectopically expressing *wnt*-5a. (A) Growth curves comparing RCC23/neo cells with RCC23/*wnt*-5a-clone 1 and RCC23/*wnt*-5a-clone 12. (B) ^{3}H-thymidine assay comparing total counts of RCC23/neo cells at confluence with RCC23/*wnt*-5a-clone 1 and RCC23/*wnt*-5a-clone 12. Error bars are the standard error of the mean.

Figure 4 - Telomerase activity of RCC23/neo cells compared to RCC23/*wnt*-5a cells. Extract from RCC23/neo cells (lanes 2-5) shows a 5-fold difference in telomerase activity as measured by the TRAP assay when compared to two different clones of RCC23/*wnt*-5a cells (lanes 6-9). Controls include (lane 1) without any extract and 0.01µg RCC23 cell extract pretreated with 200 µg/ml RNase A for 30 min at 37^{o}C (lane 10). The triangle represents increasing amounts of extract assayed (0.01, 0.05µg). The assays were done at least three times with similar results. To quantitate the levels of telomerase activity, the average optical density of first six TRAP bands after primer band was used as a ratio to ITAS band.

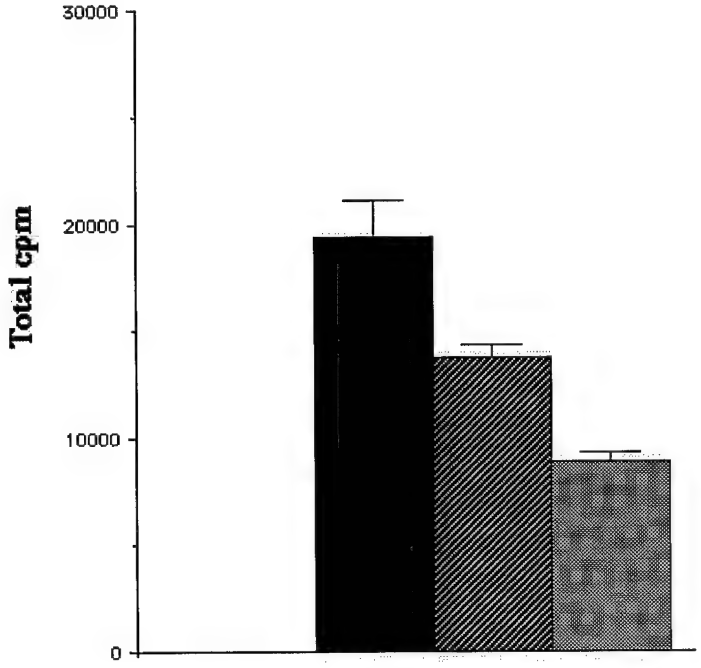

1
2
3
4
*


A
B
C

A


1250000
1000000
750000
500000
250000
0
Total number of cells
2
4
6
8
10
12
Days

B


30000
20000
10000
0
Total cpm

- ITAS
1 2 3 4 5 6 7 8 9 10
extract
RCC23 neo
clone 1
RCC23 neo
clone 2
RCC23 hwnt-5a
clone 12
RCC23 hwnt-5a
clone 1
RNase A

Figure Legends

Fig. 1. Characterization of MC-T16/wnt-5a cells. (*a*) Northern analysis of two different MC-T16 clones (lanes 3 and 4) stably expressing wnt-5a at the predicted 3200 bp size compared to MC-T16 cells (lane 2) and SV-HUC-1 cells (lane 1). The same blot was probed for GADPH for RNA loading and integrity. (*b*) Growth curves for MC-T16/wnt-5a clone 6 (○) and MC-T16/wnt-5a clone 900 (◪) cells were compared to MC-T16/neo cells (□) and parental SV-HUC-1/neo cells (✱). The results are represented as the mean +/- SE of at least three dishes.

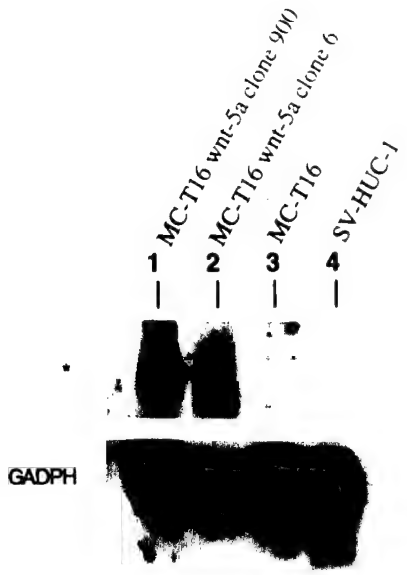
Fig. 2. Photomicrographs of confluent cells comparing MC-T16/wnt-5a cells to MC-T16/neo cells and SV-HUC-1/neo cells. (*a*) MC-T16/neo cells are refractile, elongated, and grow in nests unlike (*b*) SV-HUC-1/neo cells which are large, flat, polygonal cells. (*c, d*) MC-T16/wnt-5a cells no longer have the morphology of MC-T16/neo cells and appear somewhat similar to the phenotype of SV-HUC-1/neo cells.

Fig. 3. Tumor volumes of MC-T16/neo cells inoculated into athymic nude mice compared to SV-HUC-1/neo cells and MC-T16/wnt-5a cells. No tumor growth occurred in 10 nude mice inoculated with SV-HUC-1/neo cells (✱), nor in 10 nude mice inoculated with MC-T16/wnt-5a-clone 6 cells (○). After a lag period of 4 weeks, tumors began to grow slowly in mice inoculated with MC-T16/neo cells (□) which then rapidly accelerated after three months. This is compared to tumor development of MC-T16/wnt-5a-clone 900 cells (◪) which had a lag time of 6 weeks before the onset of tumor formation. These tumors also grew slowly and at three months stopped growing. The results are represented as the mean tumor volume +/- SE.

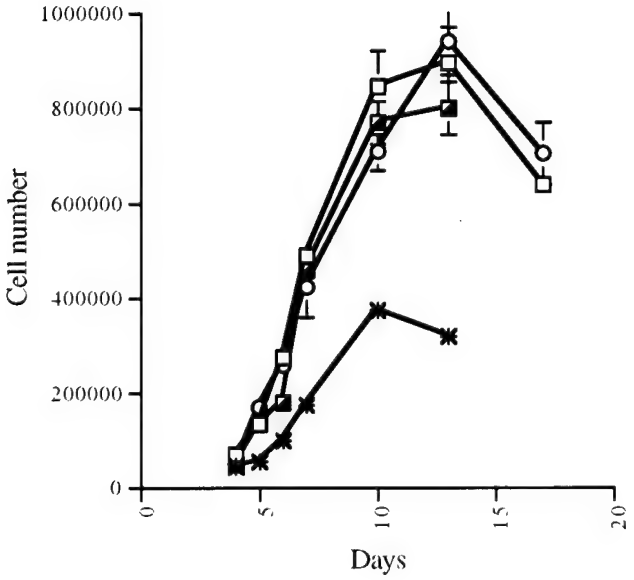
Fig. 4. Photomicrographs of tumors removed from nude mice to compare histopathology. (*a*) Tumor formed as a result of inoculation of MC-T16/wnt-5a-clone 900 cells has extensive cell death compared (*b*) to a similar size MC-T16/neo derived tumor which has no cell death. (*c*) Higher magnification of the tumor derived from MC-T16/wnt-5a-clone 900 cells demonstrates little stroma formation compared to a (*d*) MC-T16/neo cell derived tumor with extensive stromal development.

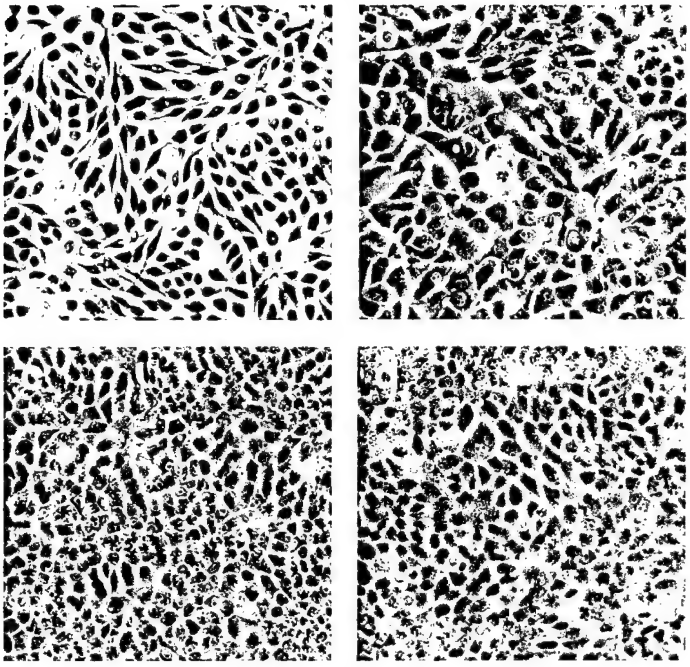
Fig. 5. Telomerase activity of MC-T16/neo cells compared to MC-T16/wnt-5a cells and SV-HUC-1/neo parental cells. Extract from SV-HUC-1/neo cells (lanes 2-4) shows no difference in telomerase activity as measured by the TRAP assay when compared to MC-T16/neo cells (lanes 5-7), MC-T16/wnt-5a-clone 6 (lanes 8-10), or MC-T16/wnt-5a-clone 900 (lanes 11-13). Controls include (lane 1), tube without any extract, (lane 14), 0.1μg SV-HUC-1 cell extract pretreated with 200 μg/ml RNase A for 30 min at 37°C, and (lane 15), positive assay control with 2 μl of TSR8 template. The triangle represents increasing amounts of extract assayed (0.01, 0.05, 0.1 μg). The assays were done at least three times with similar results.

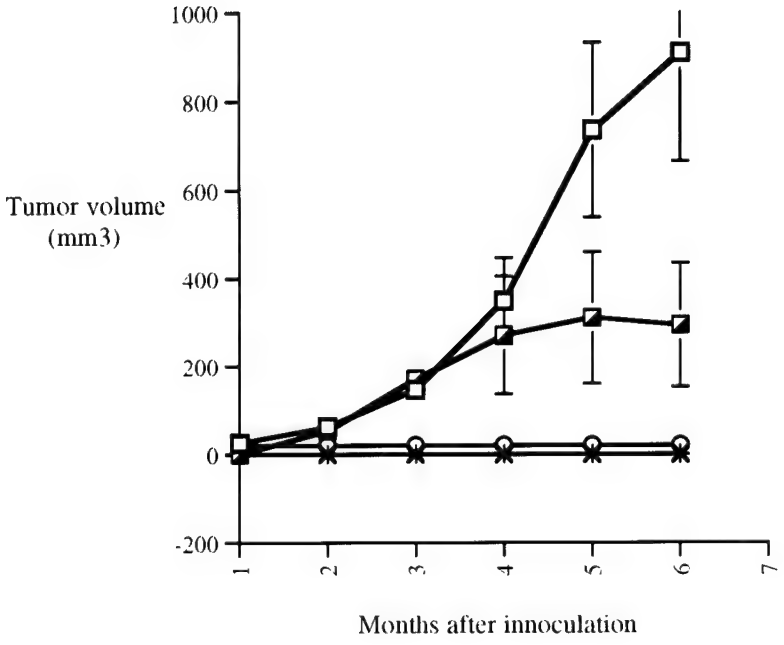
a

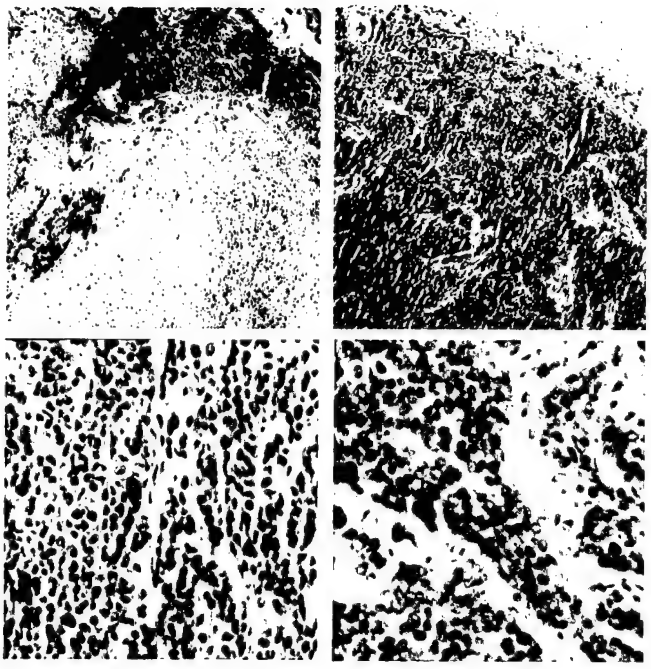

1 MC-T16 wnt-5a clone 900
2 MC-T16 wnt-5a clone 6
3 MC-T16
4 SV-HUC-1
GADPH

b

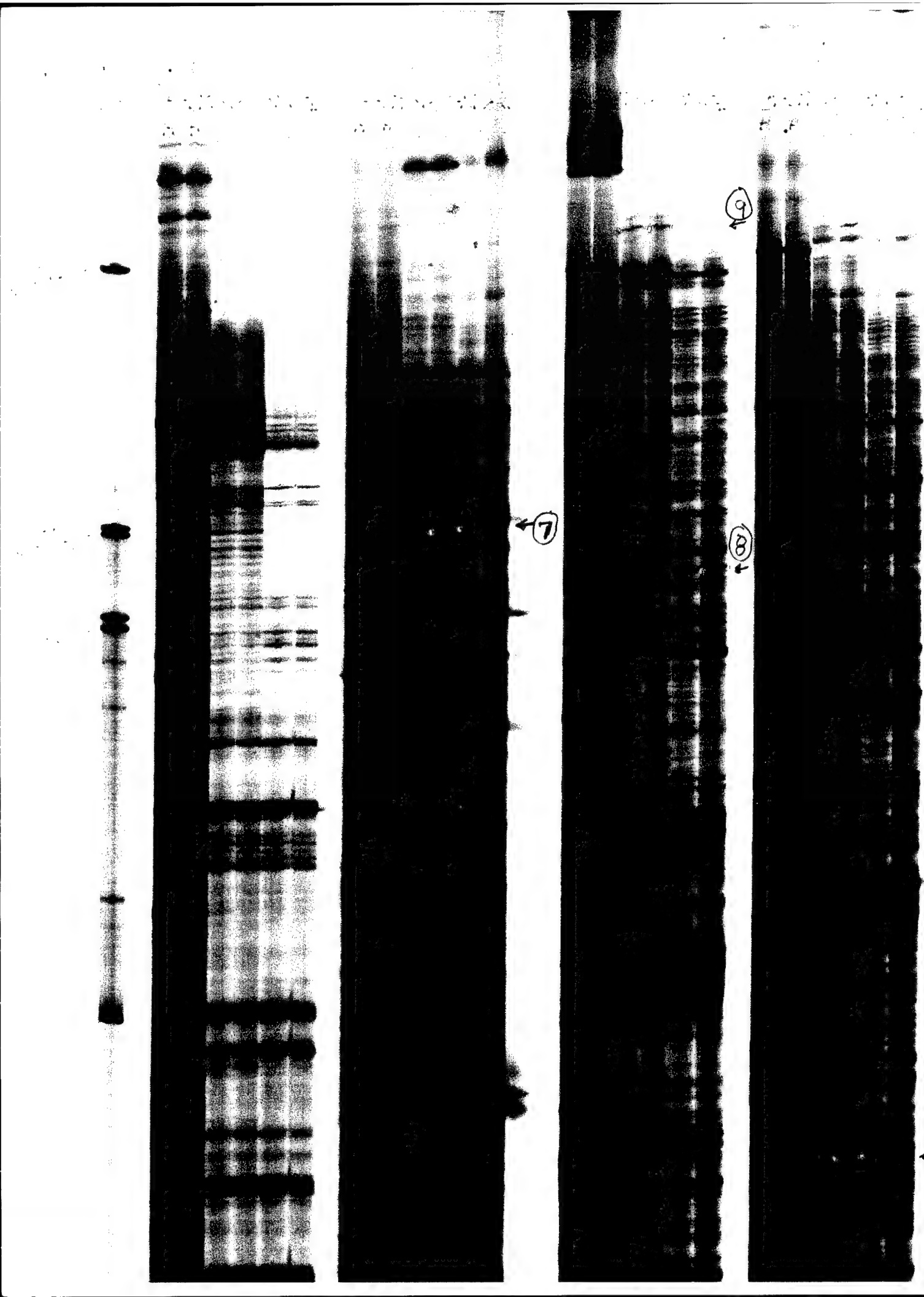

1000000
800000
600000
400000
200000
0
Cell number
0
5
10
15
20
Days




Tumor volume
(mm3)
1000
800
600
400
200
0
-200
1
2
3
4
5
6
7
Months after innoculation



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
— ITAS
extract
SV-HUC-1
MC-T16
MC-T16/wnt-5a clone 6
MC-T16/wnt-5a clone 900
+ RNase A
TSR8


9
7
8
10

EXPERIMENTAL CELL RESEARCH 241, 134–141 (1998)
ARTICLE NO. EX984030

Antisense *wnt*-5a Mimics *wnt*-1-Mediated C57MG Mammary Epithelial Cell Transformation

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Division of Plastic Surgery, Department of Surgery, Pennsylvania State University, Milton S. Hershey Medical Center, Hershey, Pennsylvania 17033

The disruption of the normal expression of *wnt*-5a in cell lines and in tumors is becoming increasingly recognized as important in cell transformation and tumorigenesis. For example, in endometrial cancer *wnt*-5a is downregulated compared to normal tissue. Our laboratory has recently found that the ectopic expression of *wnt*-5a in human RCC23 renal carcinoma cells missing *wnt*-5a gene expression suppresses *in vitro* cell growth and telomerase enzyme activity. Furthermore, ectopic *wnt*-5a in MC-T16 uroepithelial cancer cells missing the region of chromosome 3p where *wnt*-5a has been mapped reverts uroepithelial cell tumorigenesis in athymic nude mice. These studies were based upon the previous finding that *wnt*-1 and *wnt*-2 transform C57MG mammary epithelial cells by downregulating the endogenous expression of *wnt*-5a. We now report that transfecting C57MG cells with a mammalian expression vector carrying antisense *wnt*-5a results in a cell phenotype that mimics cell transformation by ectopic *wnt*-1 or *wnt*-2. Correspondingly, *wnt*-1-transformed cells are partially reverted in the presence of ectopic *wnt*-5a. We conclude from this that *wnt*-5a is an important regulator of cell growth and differentiation and its loss of expression leads to cell transformation.

INTRODUCTION

Wnt genes are members of a highly conserved family of growth factors which enter the secretory pathway and are thought to be involved in receptor-mediated signaling important in cell growth and differentiation [1, 2]. Although it is unclear how *wnt* peptides function, it is now recognized by different biologic assays that at least two functional classes of *wnt*-genes exist [3–5]. For example, some *wnt*s including *wnt*-1 and *wnt*-2 are capable of cell transformation and tumorigenesis while other *wnt*s including *wnt*-5a and *wnt*-4 are not [4, 6–8]. This is particularly well characterized in C57MG mouse mammary epithelial cells [9] that transform in the presence of ectopic *wnt*-1 or *wnt*-2 but not *wnt*-5a or *wnt*-4, which are endogenously expressed in this cell line [10]. It has been speculated that *wnt*-1 or *wnt*-2 transform C57MG cells by utilizing the endogenous *wnt* pathways, but this is highly unlikely considering that overexpression of *wnt*-5a or *wnt*-4 is not transforming [4, 8].

Wnt-gene family members have become increasingly recognized as important to the development of human tumorigenesis [11]. *Wnt*-2, 3a, 4, 5a, and 7b all have been shown to be differentially expressed when comparing normal breast tissue to benign or malignant breast cancer lesions [12–14]. There is also evidence for the involvement of the *wnt*-2 in human colorectal cancer [15]. Furthermore, *wnt*-2 has been reported to undergo compartmental switching in expression from the stromal to epithelial expression in human breast tumors [16]. More recently, *wnt*-7a has been isolated and found to likely play a role in cell transformation [17], and several *wnt*s including *wnt*-10b have been implicated in endometrial carcinoma [18] and breast cancer [19]. We have been particularly interested in the role of *wnt*-5a in human tumorigenesis. Our laboratory has found that the ectopic expression of *wnt*-5a in RCC23 renal carcinoma cells missing *wnt*-5a gene expression suppresses *in vitro* cell growth and telomerase activity (Olson *et al.*, in press). We have also reported that ectopic *wnt*-5a in MC-T16 bladder cancer cells reverts uroepithelial cell tumorigenesis in athymic nude mice [20]. These studies indicate that *wnt*-5a is a novel previously undescribed tumor suppressor gene the loss of which allows for cell transformation. This notion is supported where it was found that in endometrial cancer *wnt*-5a is downregulated compared to normal tissue [18].

Recently it was proposed that *wnt*-1 or *wnt*-2 transform C57MG cells by disruption of the normal expression of endogenous *wnt*-5a and *wnt*-4. This was based on the observation that both *wnt*-5a and *wnt*-4 are

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0014-4827/98 $25.00

downregulated in the presence of *wnt*-1 or *wnt*-2 [4]. It is hypothesized that *wnt*-5a and/or *wnt*-4 maintain normal cell growth, the disregulation of which can lead to cell transformation. For example, downregulation of *wnt*-5a to undetectable levels occurs in C57MG cells transformed by *neu* T (c-erbB2). The purpose of the present study was to directly test the hypothesis using antisense technology that loss of normal *wnt*-5a gene expression in C57MG cells would lead to cell transformation in a manner similar to overexpression of *wnt*-1 or *wnt*-2. The results indicate that C57MG cells transfected with antisense *wnt*-5a become transformed and mimic the *wnt*-1-transformed phenotype. We conclude from this that *wnt*-5a is an important growth regulator of cell growth and differentiation and its loss of expression leads to cell transformation. *Wnt*-1 cell transformation, therefore, is likely a result of the disruption of endogenous *wnt*-5a the expression of which is necessary for normal growth control.

MATERIALS AND METHODS

Cell lines. The mammalian expression vector pRSV was blunt ligated to the mouse *wnt*-5a cDNA full-length clone (gift of A. McMahon, Harvard) in the antisense orientation which was confirmed by restriction enzyme mapping. The above mammalian expression vector was cotransfected with pSV2NEO (gift of Dr. J. Papkoff, Sugen, Palo Alto, CA) into C57MG mammary epithelial cells using lipofection as described previously [21]. Stable cell lines were selected and then maintained in 250 μg/ml G-418 in Dulbecco's modified medium supplemented with 5% defined bovine serum and 5% fetal bovine serum. Several clones expressing antisense *wnt*-5a constitutively in C57MG cells were screened and confirmed by Northern blot analysis using mouse *wnt*-5a sense riboprobes hybridized to 20 μg of isolated total RNA from confluent cell cultures. To determine the effect of antisense *wnt*-5a on endogenous *wnt*-5a gene expression, poly(A) RNA was isolated and 2 μg loaded onto 1.2% formaldehyde gels. The RNA blots were probed with ^{32}P-labeled *wnt*-5a antisense riboprobes and the effect on *wnt*-5a determined by densitometry using NIH Image 1.57 software.

Transformed C57MG cells expressing *wnt*-1 under the control of an RSV promoter were maintained in Dulbecco's modified Eagle (DME) medium supplemented with 5% fetal calf serum and 5% bovine calf serum (HyClone) and 250 μg/ml G-418 (Gibco). The cells were transfected with an expression vector for *wnt*-5a ligated in the sense orientation (provided by Dr. A. P. McMahon, Harvard) using lipofection as described. The cells were cotransfected with an expression vector for hygromycin (SV2HYG, kindly provided by Dr. M. Liskay, Oregon Health Sciences University), and resistant colonies were selected and expanded into cell lines. RNA was extracted and cells expressing *wnt*-5a was verified by Northern blot analysis using specific hybridization probes for *wnt*-5a and compared to those cells only transfected with SV2HYG.

Cell growth characterization. To determine the cell saturation density, population doubling time, and morphologic phenotype of C57MG/*wnt*-1 cells, C57MG/*wnt*-1/*wnt*-5a cells, and C57MG/antisense *wnt*-5a cells, cells were plated in 12-well dishes at a density of 4×10^4 cells per well. The cells were counted every day for 8 days, and the growth rate and population doubling time were determined from the logarithmic part of the growth curve. The saturation density was determined from the cell number after the cells reached confluence at 8 days. Morphology was determined by allowing the cells to grow to confluence and photographed.

RNA isolation. Total cellular RNA was isolated from dishes of confluent cells [22]. Twenty micrograms of RNA was analyzed on a 1.0% agarose formaldehyde gel followed by transfer to Hybond-N (Amersham) membrane. The membranes were UV cross-linked (Stratagene) and prehybridized at 42°C for 3–6 hours and then hybridized at 42°C overnight with full-length cDNA *wnt*-5a probes labeled by random priming with [^{32}P]dCTP using $1-2 \times 10^6$ cpm/ml. Membranes were washed at room temperature twice in 2× SSC, 1% SDS, followed by several washes in 0.1× SSC, 0.1% SDS at 55°C. Since endogenous *wnt*-5a expression is low, it was necessary to select for poly(A)$^+$ RNA using an oligo(dT) cellulose column. Two micrograms of each poly(A)$^+$ sample was analyzed on a 1.2% agarose formaldehyde gel and transferred to a Hybond-N (Amersham) membrane. After cross-linking, the membranes were prehybridized for 3–5 h at 60°C and then hybridized overnight at 62°C with ^{32}P-labeled riboprobes made with a Riboprobe system II kit from Promega. Blots were washed at room temperature twice in 2× SSC, 1% SDS, followed by several washes in 0.1× SSC, 0.1% SDS at 65°C. The prehybridization and hybridization solutions consisted of 50% formamide, 4× SSPE, 0.2 mg/ml sheared and boiled salmon sperm DNA, 2.5× Denhardt's, and 1% sodium dodecyl sulfate (SDS). The membranes were then mounted on 8 × 10 film (Kodak) with an intensifying screen and placed at −80°C up to 5 days.

Soft agar assay. In triplicate, 10^4 cells were plated in 0.35% agar (Noble) suspension using standard medium over a previously poured 0.5% agar base in 12-well dishes. G-418-selected C57MG cells were

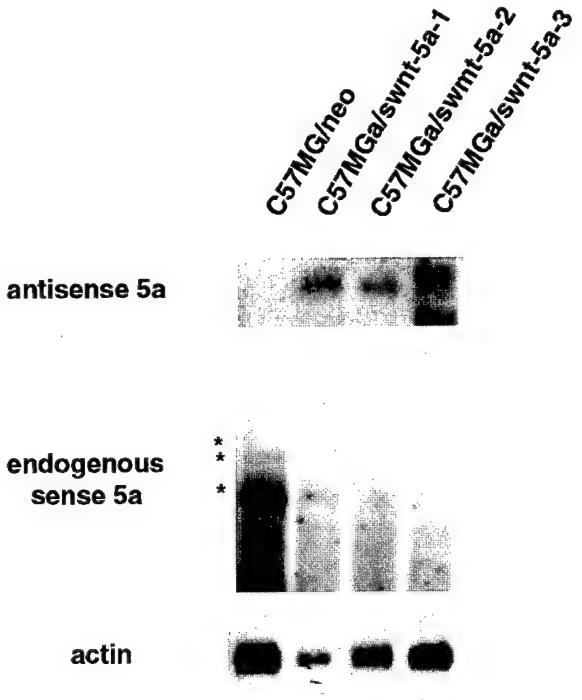

FIG. 1. Northern blot analysis of C57MG cells transfected with antisense *wnt*-5a. (Top) Three clones stably expressing antisense *wnt*-5a compared to the parental cell line (lane 1) determined by probing the blot with a *wnt*-5a sense riboprobe. (Middle) The effect of ectopic antisense *wnt*-5a on endogenous *wnt*-5a determined by probing with an antisense *wnt*-5a riboprobe. (Bottom) Middle panel blot normalized for RNA loading using an actin cDNA probe. By densitometry, endogenous *wnt*-5a decreases 6-fold in clone 1 (lanes 2), 8-fold in clone 2 (lane 3), and 12-fold in clone 3 (lane 4).

used as a negative control. C57MG cells expressing ectopic *neu* T (c-erbB2) (from Oncogene Science) were used as a positive control. Three clones of C57MG cells expressing antisense *wnt*-5a were plated for comparison. Colony formation was determined daily for 2 weeks. Medium was added to the wells as needed. Three separate soft agar assays were done and the results pooled.

[^{3}H]Thymidine incorporation. Cell lines were plated into 5-cm dishes in standard growth medium and grown to confluence with medium change every 2 days (14 days). In triplicate, 4 μCi of [^{3}H]-thymidine (methyl-^{3}H, 60–90 Ci/mmol, aqueous, ICN) was added to each dish and incubated for 2 h at 37°C. The cells were washed twice with phosphate-buffered saline (PBS) followed by addition of 2 ml ice-cold 10% trichloroacetic acid (TCA) and incubated for 30 min on ice. Cells were then washed with 10% TCA followed by addition of 2 ml of 0.1 N NaOH. The dishes were incubated at 37°C for 30 min and then neutralized with 0.2 ml of 1 N HCl. The dishes were carefully scraped and the extract added to 10 ml of scintillation fluid for counting. A fourth dish of cells was grown in parallel to determine total protein and the counts normalized to total protein. The results reflect the summary of three separate experiments.

RESULTS

RNA Expression

Cells lines were screened for antisense *wnt*-5a expression and for endogenous *wnt*-5a expression by Northern blot analysis. Three cell lines are shown in Fig. 1 (lanes 2–4) which are expressing *wnt*-5a antisense compared to the control C57MG/neo cells (lane 1). The blots were probed for actin expression to determine loading and RNA integrity (Fig. 1, bottom panel). The effect of antisense *wnt*-5a on endogenous *wnt*-5a expression was compared shown in the middle panel of Fig. 1. By densitometry, endogenous *wnt*-5a was down-

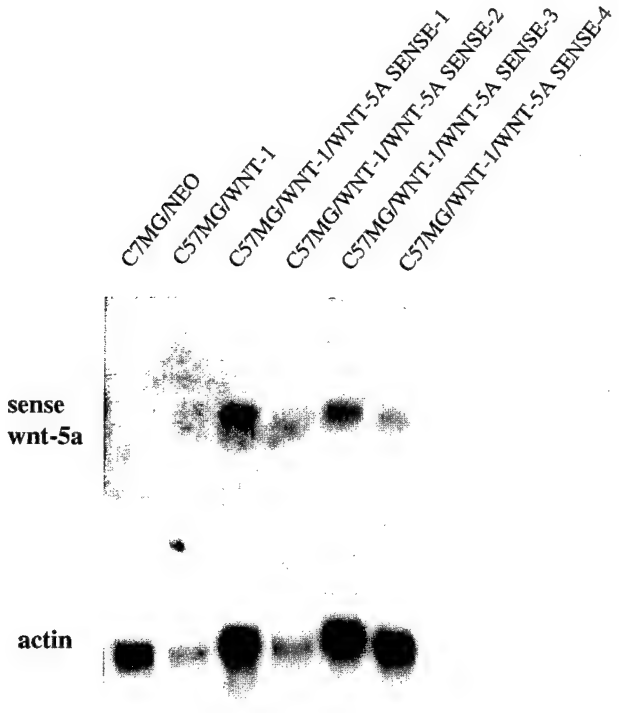

FIG. 2. Northern blot analysis of C57MG cells expressing *wnt*-1 (known to downregulate endogenous *wnt*-5a) which have been transfected with sense *wnt*-5a. Shown are C57MG parental cells (lane 1) compared to transformed C57MG/*wnt*-1 cells (lane 2). Four C57MG/*wnt*-1 clones stably expressing sense *wnt*-5a are seen in lanes 3–6. The same blot is normalized for RNA loading using an actin cDNA probe. Three clones were chosen for further analysis (lanes 3, 5, 6).

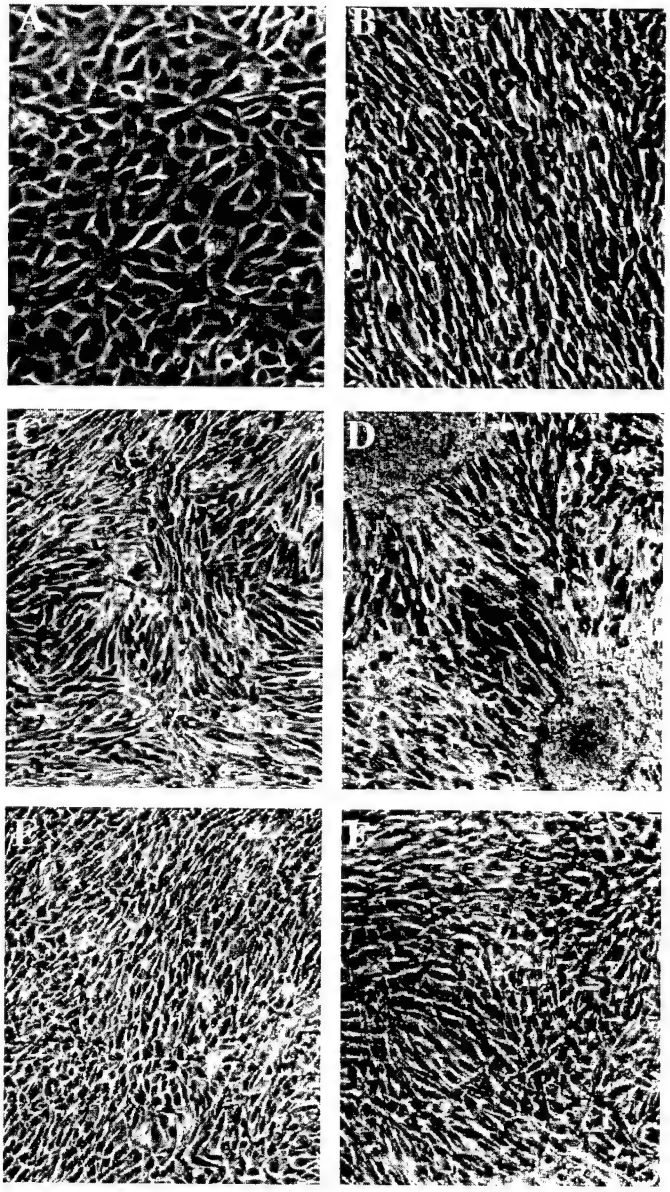

FIG. 3. Photomicrographs of three clones stably expressing antisense *wnt*-5a in C57MG cells. The parental cells (A) at confluence are compared to transformed C57MG cells expressing *wnt*-1 (B) known to downregulate endogenous *wnt*-5a. Clones expressing ectopic antisense *wnt*-5a (C–F) have a morphologic phenotype similar to that seen when the cells are transformed with *wnt*-1. The highest expressing antisense *wnt*-5a clone (clone 3) (C and D) tends to form foci at confluence.

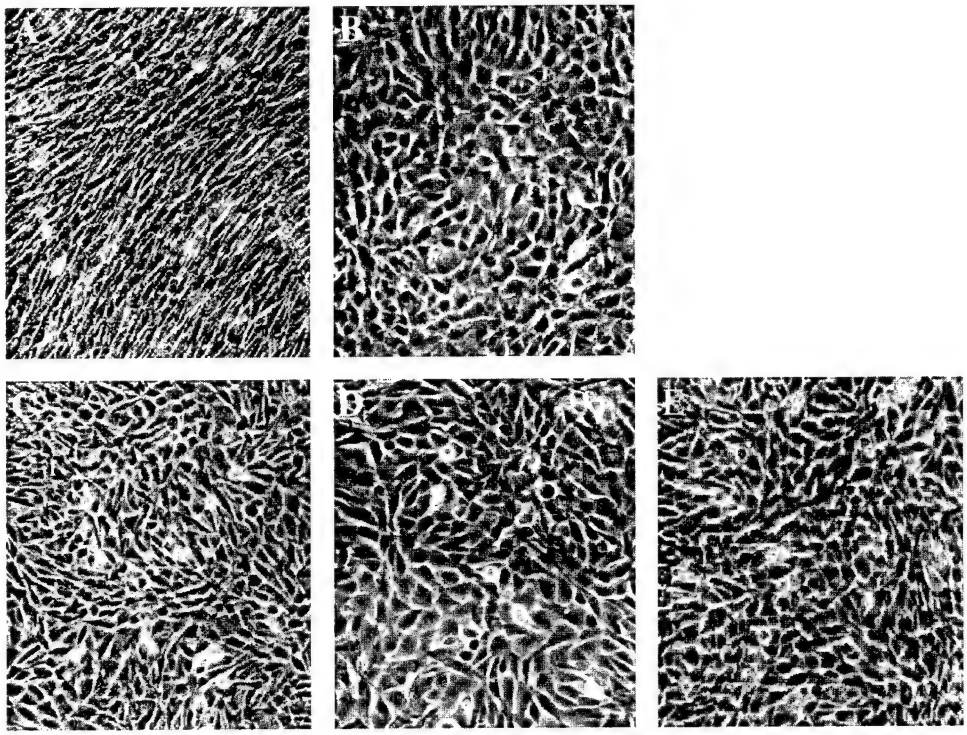

FIG. 4. Photomicrographs of three clones stably expressing sense *wnt*-5a in C57MG/*wnt*-1 cells. The parental cells (B) at confluence are compared to transformed C57MG cells expressing *wnt*-1 (A) known to downregulate endogenous *wnt*-5a. Clones expressing ectopic sense *wnt*-5a (C–E) have a morphologic phenotype similar to that seen in the parental cells suggesting that ectopic *wnt*-5a can revert the *wnt*-1-transformed cell phenotype.

regulated 6-fold in clone 1 (lane 2), 8-fold in clone 2 (lane 3), and 12-fold in clone 3 (lane 4).

Cell lines already transformed by ectopically expressed *wnt*-1 were screened for expression of transfected sense *wnt*-5a by Northern blot. Four clones are shown in Fig. 2 which were found to express *wnt*-5a (lanes 3–6) compared C57MG/*wnt*-1 cells (lane 2) and parental C57MG/neo/hyg cells (lane 1).

Morphology

C57MG cells expressing antisense *wnt*-5a and C57MG cells expressing *wnt*-1 known to downregulate endogenous *wnt*-5a (4) were grown to confluence and photographed for comparison of morphologic phenotype (Fig. 3). Little or no difference was apparent in cells transformed by *wnt*-1 (Fig. 3B) and in cells expressing antisense *wnt*-5a (Figs. 3C–3F) compared to parental C57MG/neo/hyg cells (Fig. 3A) which are flat and cuboidal. Interestingly, clone 3 which is expressing the highest level of antisense *wnt*-5a and having the most effect on the gene expression of endogenous *wnt*-5a appears highly transformed in that the cells at confluence tend to form foci (Figs. 3C and 3D).

Wnt-1-mediated transformed C57MG cells transfected with *wnt*-5a in the sense orientation (Fig. 4) revert their cell phenotype to one that appears more like the parental cells. Three different *wnt*-1-transformed clones (Figs. 4C–4E) expressing *wnt*-5a no longer appear like cells expressing ectopic *wnt*-1 (Fig. 4A) but rather look similar to C57MG/neo/hyg parental cells (Fig. 4B).

Taken together these results suggest that disregulation of endogenous *wnt*-5a gene expression disrupts normal cell growth and when ectopically expressed is capable of reverting the *wnt*-1-mediated transformed phenotype.

Growth Kinetics

Plated C57MG cells transfected with antisense *wnt*-5a were counted daily until reaching confluence. Population doubling time appeared not to be significantly altered by the expression of antisense *wnt*-5a in C57MG cells nor by the ectopic expression of sense *wnt*-5a in *wnt*-1-transformed C57MG cells (Figs. 5A and 6A and Tables 1 and 2). Yet, the saturation density of cells at confluence was significantly different. That is, C57MG cells transfected with antisense *wnt*-5a had a four- to fivefold increase in saturation density compared to the parental cells and similar to the sixfold increase seen with *wnt*-1-transformed C57MG cells.

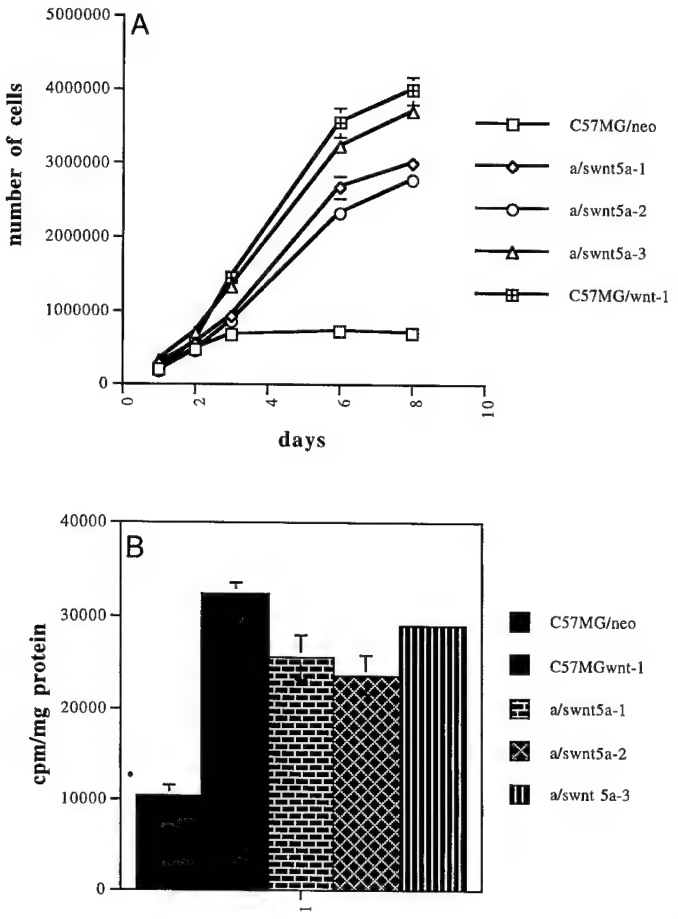

FIG. 5. Growth kinetics of C57MG cells expressing antisense *wnt*-5a indicate that saturation density at confluence for all three clones is similar to that found when the cells are transfected with *wnt*-1 but unlike that for the parental cells (A). DNA synthesis in all three clones expressing antisense *wnt*-5a is increased at confluence similar to that seen when the cells are expressing *wnt*-1 but unlike that in the parental cells (B).

When cells already transformed with ectopic *wnt*-1 are transfected with sense *wnt*-5a, the saturation density decreases one to two times compared to cells expressing only *wnt*-1. These results suggest a role for *wnt*-5a in cell growth that affects contact growth inhibition. However, C57MG cells transfected with antisense *wnt*-5a did not affect colony formation in soft agar assays (data not shown). Since *wnt*-1-mediated cell transformation does not result in anchorage-independent growth, it would not be expected that the loss of endogenous *wnt*-5a by the expression of antisense *wnt*-5a would result in anchorage-independent growth.

[^{3}*H*]*Thymidine Incorporation*

C57MG cells transfected with antisense *wnt*-5a incorporated two to three times more labeled thymidine at confluence when compared to parental cells which was similar to that found when C57MG cells are transformed by *wnt*-1 (Fig 5B). When *wnt*-1-transformed cells were transfected with *wnt*-5a, DNA synthesis at confluence significantly decreases to a level approaching that found in the parental cell line (Fig. 6B). This supports the notion that *wnt*-5a expression is important for C57MG cell growth and that loss of expression compromises growth control.

DISCUSSION

We were interested in testing the notion that *wnt*-1 transforms C57MG mammary epithelial cells by downregulating the endogenous expression of *wnt*-5a [4]. This was directly tested by transfecting C57MG cells with a mammalian expression vector carrying antisense *wnt*-5a. In this report we have shown that when endogenous *wnt*-5a gene expression in C57MG cells is downregulated by the ectopic expression of antisense *wnt*-5a, the cells transform in a manner similar to that

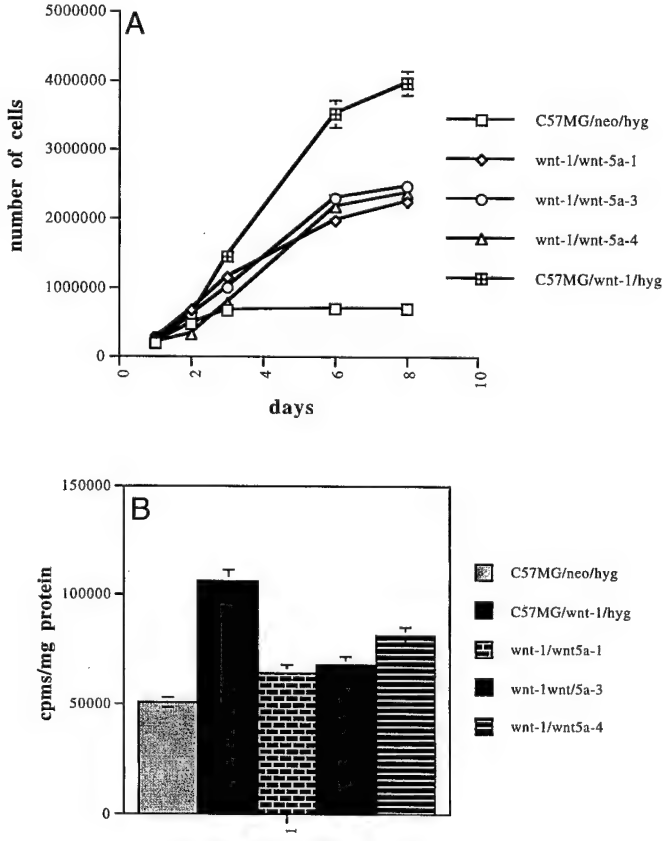

FIG. 6. Growth kinetics of C57MG/*wnt*-1-transformed cells expressing sense *wnt*-5a indicate that the saturation density at confluence for all three clones has decreased indicating a partial reversion of cell growth when compared to parental cells (A). DNA synthesis in all three clones expressing sense *wnt*-5a is decreased at confluence similar to that seen in the parental cells (B).

TABLE 1

Growth Characteristics of C57MG Mammary Epithelial Cells Transfected with Antisense *wnt*-5a

Cell line	Population doubling time (h)	Saturation density (10^6 cells)	[^{3}H]Thymidine (cpm/mg protein)
C57MG/neo (−)	35	0.69	50,544
C57MG/antisense *wnt*-5a-1	35	2.99	68,054
C57MG/antisense *wnt*-5a-2	31	2.77	64,321
C57MG/antisense *wnt*-5a-3	36	3.68	81,160
C57MG/*wnt*-1 (+)	30	3.98	105,927

which occurs with ectopic *wnt*-1 expression (known to downregulate *wnt*-5a). Furthermore, we were able to revert the *wnt*-1-transformed phenotype in C57MG cells by overexpressing sense *wnt*-5a. This suggests that *wnt*-5a controls normal growth control in this cell line which supports the proposed hypothesis that *wnt*-1 transforms C57MG cells by disregulating endogenous *wnt*-5a gene expression. Correspondingly, *wnt*-5a appears to be capable of antagonizing *wnt*-1 function in C57MG cells.

The antagonistic relationship between *wnt*-1 and *wnt*-5a and possibly between other *wnt*-gene family members has been reported previously [23]. In C57MG cells, ectopic *wnt*-1 is highly transforming while overexpression of *wnt*-5a is not [4, 8]. Biochemical analysis comparing properties of various *wnt* genes transiently expressed in COS cells reveals distinct differences between *wnt*-5a and *wnt*-1 [24]. In *Xenopus* frog assays, *wnt*-1 but not *wnt*-5a is able to duplicate the embryonic axis and enhance gap junctional communication [25, 26]. This has been elucidated further in that *wnt*-5a can directly antagonize the functional activity of *wnt*-1 which has led to the functional separation of these two *wnt*-gene family members to be categorized as part of two distinct functional classes of genes [4, 5, 23]. Other important distinctions have been made between *wnt*-1 and *wnt*-5a activities that relate to tumorigenesis. *Wnt*-1 enhances telomerase activity while *wnt*-5a suppresses telomerase activity [27] (Olson *et al.*, in press). *Wnt*-1 enhances Ca^{2+}-dependent cell adhesion possibly through cadherin/catenin binding [28] while *wnt*-5a represses Ca^{2+} signaling in embryos [29]. The data presented in this report also show that ectopic *wnt*-5a can antagonize *wnt*-1 transformation in mammalian cells. This supports the proposition that *wnt*-5a and *wnt*-1 are members of distinct functional *wnt*-gene classes, but also support the idea that regulatory interaction occurs between these two *wnt*-gene classes.

The disruption of the normal gene expression of *wnt*-5a appears to be important in human cell transformation and in tumorigenesis. In human malignant cell lines *wnt*-5a RNA expression is low or is nondetectable [12]. In breast cancer, *wnt*-5a expression decreases compared to benign tumors [12] and in endometrial carcinoma *wnt*-5a is also downregulated [18]. This suggests that loss of *wnt*-5a is important in the progression of events that lead to the development of cancer. This idea has been tested recently by the overexpression of *wnt*-5a in MC-T16 malignant uroepithelial cells missing *wnt*-5a which reverts the cell phenotype and represses tumorigenesis in nude mice [20]. How this occurs is unknown, but the MC-T16 cells expressing *wnt*-5a are no longer able to migrate across wounds created in monolayer cultures suggesting compromise of *wnt*-5a on mechanisms involving cell migration and cell adhesion (Olson, unpublished observations). This is supported by other studies which demonstrate that ectopic *wnt*-5a is able to prevent cell migration [30, 31] and is upregulated in monolayer cultures when cells reach confluence [32]. It has been suggested that upregulation of *wnt*-5a gene expression controls normal cell growth by preventing migration at confluence [32].

TABLE 2

Growth Characteristics of C57MG/*wnt*-1 Mammary Epithelial Cells Transfected with Sense *wnt*-5a

Cell line	Population doubling time (h)	Saturation density (10^6 cells)	[^{3}H]Thymidine (cpm/mg protein)
C57MG/neo/hyg (−)	35	0.69	10,248
C57MG/*wnt*-1/sense*wnt*-5a-1	28	2.24	23,600
C57MG/*wnt*-1/sense*wnt*-5a-3	38	2.49	28,801
C57MG/*wnt*-1/sense*wnt*-5a-4	30	2.38	25,464
C57MG/*wnt*-1 (+)	30	3.98	32,269

This may explain the paradoxical upregulation in growing benign and malignant tumors [12, 14]. That is, *wnt*-5a may be upregulated in an attempt to limit cell movement at a time when tumor cells are rapidly growing. In this study, C57MG cells transfected with antisense *wnt*-5a continue to grow after reaching confluence suggesting the loss of contact growth inhibition. Interestingly, soft agar colony formation did not occur in the cell lines expressing antisense *wnt*-5a. This is not surprising since *wnt*-1-transformed cells are not tumorigenic and do not grow in soft agar [8]. However, we have previously found that MC-T16 human bladder cancer cells, which have loss of heterozygosity of chromosome 3p13-21 [33] where *wnt*-5a has been mapped [34], when transfected with *wnt*-5a have reestablished contact-dependent growth inhibition determined by soft agar assays [20]. Furthermore, C57MG cells transfected with *neu* T which downregulates *wnt*-5a to undetectable levels form colonies in soft agar assays [4]. These data suggest that the manner in which loss of *wnt*-5a allows for cell transformation is by affecting mechanisms which influence cell migration and contact growth inhibition.

These studies indicate that *wnt*-5a is required for normal growth of C57MG cells and likely for other mammalian cell types and that loss of *wnt*-5a expression allows for cell transformation to occur. This has important implications for understanding the manner in which known *wnt*-oncogenes and growth factors transform cells. In this context *wnt*-5a may be characterized as a *wnt*-anti-oncogene. This especially relevant to the multistep progression of the development of human cancers, at least one of which is known to revert in the presence of ectopic *wnt*-5a [20].

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Ectopic Expression of *wnt*-5a in Human Renal Cell Carcinoma Cells Suppresses in vitro Growth and Telomerase Activity

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Abstract

Loss of genes located on chromosome 3p has been reported in many different types of human cancers, including renal cell carcinoma. Previous studies using a nontumorigenic human renal cell carcinoma cell line (RCC23) established from a stage III nonpapillary carcinoma with a loss of heterozygosity on 3p showed that microcell hybrids containing an introduced intact chromosome 3 resulted in a more differentiated phenotype including restored cellular senescence and repression of telomerase activity. Human *wnt*-5a has been cloned and mapped to chromosome 3p14-21. We have stably transfected human *wnt*-5a into RCC23 cells which results in in vitro growth suppression and repression of telomerase activity in a manner similar to that found in microcell hybrids containing an introduced intact chromosome 3.

Key Words
Anti-oncogene
Kidney cancer
Differentiation
Chromosome 3p deletion

Introduction

Deletions of a gene or genes located on chromosome 3p have been reported in many different types of carcinomas [1–4]. Although the biological significance of 3p deletions is uncertain, there is a close correlation between 3p deletions and malignant transformation of several epithelial cell types, suggesting the loss of function of at least one gene located on chromosome 3p which controls normal cell growth and differentiation [5, 6]. Cytogenetic analysis demonstrates that the region 3p11-p25 carries one or more tumor suppressor gene(s) [7–12].

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In human renal cell carcinoma, loss of alleles and cytogenetic aberrations have been well documented. For example, up to 96% of nonpapillary forms of renal cell carcinomas have been shown to have a nonrandom loss of chromosome 3p [13, 14]. Furthermore, loss of heterozygosity (LOH), determined by comparative genomic hybridization, occurs consistently in renal cell carcinoma, suggesting the loss of one or more tumor suppressor genes which likely play a significant role in renal cell carcinogenesis [15, 16]. Efforts have been made recently to map specific regions of chromosome 3p where suppressor genes may be localized [17, 18]. One suppressor gene has been mapped to 3p13-14.2 and another distal to 3p21.3 in renal cell carcinoma [19]. Another region encompassing 3p12-p14 has been found to dramatically alter tumor growth in nude mice when a fragment containing this region was introduced into a highly malignant nonpapillary renal cell carcinoma cell line [20]. This region includes a translocation breakpoint in familial renal cell carcinoma [21].

We have been investigating whether human *wnt*-5a is a growth-regulating gene the loss of which is important in the progression of renal cell carcinoma. Olson and Papkoff [22] previously found that *wnt*-5a endogenously expressed in mouse mammary epithelial cells may control normal cell growth. That is, loss of normal gene expression of *wnt*-5a in the presence of *wnt*-1, *wnt*-2, or *neu* T correlates with cell transformation. Furthermore, hepatocyte growth factor (HPG), a poor prognostic indicator in some cancers, has also been found to downregulate *wnt*-5a gene expression [23]. This has recently been extended directly using antisense *wnt*-5a which results in the loss of normal *wnt*-5a gene expression and leads to cell transformation [Olson et al., unpubl. data]. Human *wnt*-5a has been cloned and mapped to chromosome 3p14-p21 [24, 25]. Recently, we found that *wnt*-5a expressed in uroepithelial cancer cells with an LOH for chromosome 3p13-p21.2 results in reversion of uroepithelial cell tumorigenesis in athymic nude mice [26].

Currently, we are pursuing whether ectopic *wnt*-5a is capable of reestablishing normal cell growth in a nontumorigenic human renal cell carcinoma cell line (RCC23) established from a stage III nonpapillary carcinoma with an LOH on 3p [27]. Previous studies using RCC23 cells showed that microcell hybrids containing an introduced intact chromosome 3 resulted in a significant reduction in growth rate, saturation density, and altered morphologic phenotype compared to the parental cancer cells [27]. Furthermore, it was later demonstrated that the introduction of chromosome 3 also restored cellular senescence which was associated with repression of telomerase activity [28].

In this report we have stably transfected human *wnt*-5a into RCC23 cells which results in in vitro growth suppression and repression of telomerase activity in a manner similar to that found in microcell hybrids containing an introduced intact chromosome 3. This suggests that human *wnt*-5a regulates cell growth and may function as a tumor suppressor gene. *wnt*-5a may be one of the suppressor genes deleted or rearranged on chromosome 3p in renal cell carcinoma.

Material and Methods

Transfection and Cell Characterization

Full-length human *wnt*-5a cDNA (clone T11) [24] was the kind gift of Dr. Renato Iozzo (Jefferson Medical College, Philadelphia, Pa., USA). The cDNA was subcloned into pRSV (Dr. Jackie Papkoff, Sugen, Redwood City, Calif., USA) and orientation determined by restriction analysis. RCC23 renal cell carcinoma cells characterized previously [27] were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin.

Cells were grown to 80% confluence before each passage. For gene transfection, passage 18 cells were grown to 50% confluence and the medium exchanged for low serum medium (Optimem, Gibco). Using 90 µl liposomes (Lipofectin, Gibco) mixed with 10 µg of pRSV*wnt*-5a and/or pSV2neo in a total volume of 150 µl, the cells were transfected overnight at 37°C in 5% CO_2. The medium was replaced and the cells grown overnight in medium with 1% FBS without the addition of Geneticin (G-418, Gibco). The cells were then selected in medium supplemented with 800 µg/ml G-418. Individual clones were isolated, resistant colonies expanded into cell lines, and maintained in medium supplemented with 250 µg/ml G-418 for eventual RNA extraction to determine gene expression of *wnt*-5a. To determine the cell saturation density, population doubling time, and morphologic phenotype of RCC23/neo cells and RCC23/*wnt*-5a cells, cells were plated in 12-well dishes at a density of 4×10^4 cells per well. The cells were counted every day for 10 days and the growth rate and population doubling time determined from the logarithmic part of the growth curve. The saturation density was determined from the cell number after the cells reached confluence at 10 days. Morphology was determined by allowing the cells to grow to confluence and photographed.

RNA Isolation and Analysis

Total cellular RNA was isolated from dishes of confluent cells [29]. Twenty micrograms of RNA were analyzed on a 1.0% agarose formaldehyde gel followed by transfer to Hybond-N (Amersham) membrane. The membranes were UV cross-linked (Stratagene) and prehybridized at 42°C for 3–6 h, and then hybridized at 42°C overnight with full-length *wnt*-5a c-DNA probes labeled by random priming with [^{32}P]dCTP using $1–2 \times 10^6$ cpm/ml. The prehybridization and hybridization solutions consisted of 50% formamide, 4 × SSPE, 0.2 mg/ml sheared and boiled salmon sperm DNA, 2.5 × Denhardt's, and 1% sodium dodecyl sulfate (SDS). Membranes were washed at room temperature twice in 2 × SSC, 1% SDS, followed by several washes in 0.1 × SSC, 0.1% SDS at 55°C. The membranes were then mounted on 8 × 10 film (Kodak) with an intensifying screen and placed at –80°C up to 5 days.

^{3}H-Thymidine Incorporation

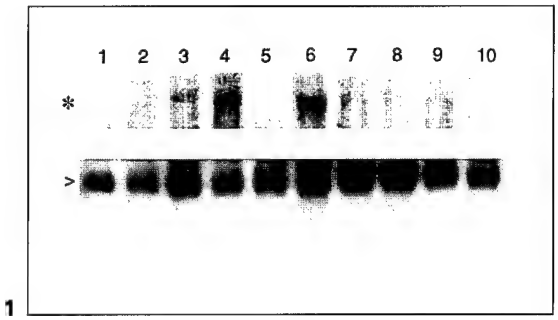
Cell lines were plated into 5-cm dishes in standard growth medium and grown to confluence with medium change every 2 days (10 days). In triplicate, 4 µCi of ^{3}H-thymidine (methyl ^{3}H, 60–90 Ci/mmol, aqueous, ICN) was added to each dish and incubated for 2 h at 37°C. The cells were washed twice with phosphate-buffered saline (PBS) followed by addition of 2 ml ice cold 10% trichloroacetic acid (TCA) and incubated for 30 min on ice. Cells were then washed with 10% TCA followed by addition of 2 ml of 0.1 *N* NaOH. The dishes were incubated at 37°C for 30 min and then neutralized with 0.2 ml of 1 *N* HCl. The dishes were carefully scraped and the extract added to 10 ml of scintillation fluid for counting. A fourth dish of cells was grown in parallel to determine total protein and the counts normalized to total protein. The results reflect the summary of three separate experiments.

Telomere Length

Southern blot analysis of telomere length was done on RCC23/neo parental cells and on two clones (clones 1 and 12) of RCC23 cells transfected with and expressing *wnt*-5a. DNA was extracted from primary cultured cells at passage 18, *Hin*fI digested and 5 µg loaded and separated by electrophoresis on a 0.7% agarose gel. This was transferred to a nylon membrane, and hybridized to the ^{32}P-labeled telomeric probe $(TTAGGG)_4$ in 6 × SSPE-1% SDS at 50°C. Membrane washings were in 6 × SSC–0.1% SDS at 50°C. The mean length of the terminal restriction fragment (TRF) was determined by densitometry.

Telomerase Assay

Two different RCC23/neo cell lines were compared for telomerase activity to two different RCC23/*wnt*-5a cell lines. Subconfluent cultures were used to prepare the detergent 3-[(cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) extracts [30]. Telomerase enzyme activity was measured by using a PCR-based telomeric repeat amplification protocol (TRAP) kit from Oncor, Inc., as per manufacturer's instructions. Each reaction product was amplified in the presence of an internal TRAP assay standard (ITAS, 36 bp). The TRAP reaction products were separated by 10% PAGE, dried, and autoradiographed. The basal levels of telomerase activity (ladder formation) was measured by serial dilution of the protein extracts, and an appropriate range of protein concentration selected that produced a linear response as described [31]. Each set of TRAP assays included control reaction tubes without any extract, and extracts treated with RNase A (200 µg/ml). To quantitate the levels of telomerase activity, the average optical density of the first six TRAP bands after primer band was used as a ratio to ITAS band.



1

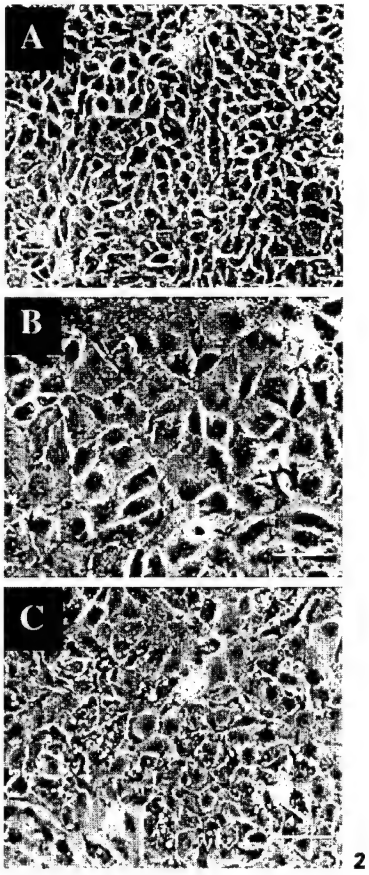


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Fig. 1. Northern analysis of RCC23 cells (lane 1) and RCC23/neo cells (lane 2) compared to RCC23 cells transfected with *wnt*-5a (lanes 3–10). The same blot was probed with cyclophilin (>) for RNA loading and integrity. The asterisk (*) indicates the *wnt*-5a signal. RCC23/*wnt*-5a clone 1 (lane 4) and RCC23/*wnt*-5a clone 12 (lane 6) were selected for further analysis.

Fig. 2. Photomicrographs of confluent cells comparing two clones of stably expressing RCC23/*wnt*-5a cells to RCC23/neo cells. **A** RCC23/neo cells are refractile, tightly packed with polygonal morphology unlike RCC23/*wnt*-5a clone 1 cells (**B**) which are large and flat, and RCC23/*wnt*-5a clone 12 cells (**C**) which in addition to being large and flat have vacuolarization. Bars = 60 μm.

Results

Analysis of RCC23 Cells Transfected with wnt-*5a*

Renal cell carcinoma cells (RCC23) were transfected with full-length human *wnt*-5a using liposomes (Lipofectin, Gibco). Clones were stably selected in G-418, isolated, and expanded into stable cell lines. Northern analysis using a random primed *wnt*-5a cDNA ^{32}P labeled probe demonstrated expression of the expected 3.2-kb size RNA in several clonally expanded cell lines (fig. 1, lanes 3–10) compared to the parental cell lines (lanes 1 and 2). Two clones with high *wnt*-5a expression (lanes 4 and 6) were selected for comparison to the RCC23 cell line resistant to G-418 expression (lane 2). RCC23 cells characteristically have polygonal morphology which at confluence become tightly packed (fig. 2A). However, when two different clones of RCC23 cells are ectopically expressing *wnt*-5a, the cells at confluence become flattened and pleiomorphic, without becoming tightly packed (fig. 2B, C). Interestingly, clone 12 (fig. 2C) cells have extensive cytoplasmic vacuolarization which could represent cellular senescence. These findings suggest that RCC23 cells ectopically expressing *wnt*-5a alter the cell morphologic phenotype to one that is better differentiated.

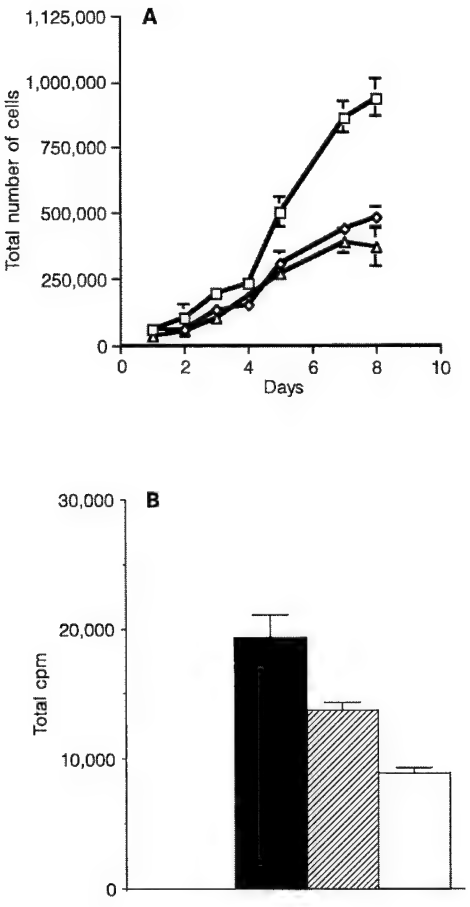

Fig. 3. Growth kinetics of RCC23 cells compared to RCC23 cells ectopically expressing *wnt*-5a. **A** Growth curves comparing RCC23/neo cells (□) with RCC23/*wnt*-5a-clone 1 (◇) and RCC23/*wnt*-5a-clone 12 (△). **B** ^{3}H-thymidine assay comparing total counts of RCC23/neo cells (■) at confluence with RCC23/*wnt*-5a-clone 1 (▨) and RCC23/*wnt*-5a-clone 12 (□). Error bars are the standard error of the mean.

In vitro Growth Assays

The growth properties were compared between the parental cells and cells expressing *wnt*-5a. As shown in figure 3A, the growth rate of the RCC23 cells expressing *wnt*-5a was remarkably slower than that for the parental cells. The population doubling time of RCC23/neo cells was 36 h while the doubling time for RCC23/*wnt*-5a-clone 12 cells was 72 and 60 h for RCC23/*wnt*-5a-clone 1. Cell saturation density at confluence corresponded with growth rate. That is, RCC23 parental cells had a saturation density of 9.4×10^5 at 10 days post-passage, while both RCC23 clones expressing *wnt*-5a had saturation densities of 4.8×10^5 and 3.7×10^5. These results support the notion that the expression of *wnt*-5a alters cell growth. This is further supported by the incorporation of ^{3}H-thymidine at confluence (fig. 3B). Both clones of RCC23 cells expressing *wnt*-5a incorporated significantly less ^{3}H-thymidine at confluence than the parental cell line. That is, clone 12 incorporated 8,942 ± 355 cpm and clone 1 incorporated 13,771 ± 571 cpm compared to the parental cell line which incorporated 19,341 ± 1,745 cpm.

Telomere Length and Telomerase Assay

Telomere lengths were determined by hybridization of the $(TTAGGG)_4$ probe to *Hin*fI-digested DNA from parental RCC23 G-418 selected cells compared to RCC23 cells expressing *wnt*-5a. The TRF values for all three cell lines were not significantly different (data not shown). Previous studies have indicated repression of telomerase activity in RCC23 microcell hybrids introduced with chromosome 3 [28]. The process of transformation can be associated with the activation of telomerase, a ribonucleoprotein enzyme complex that adds telomeric repeats (hexanucleotide 5′-TTAGGG-3′) to the ends of replicating chromosomes or telomeres. In addition, *wnt*-1 (which downregulates the expression of *wnt*-5a [22]) has been recently shown to regulate telomerase activity [32]. We compared telomerase activity between two different clones of G-418-resistant clones of

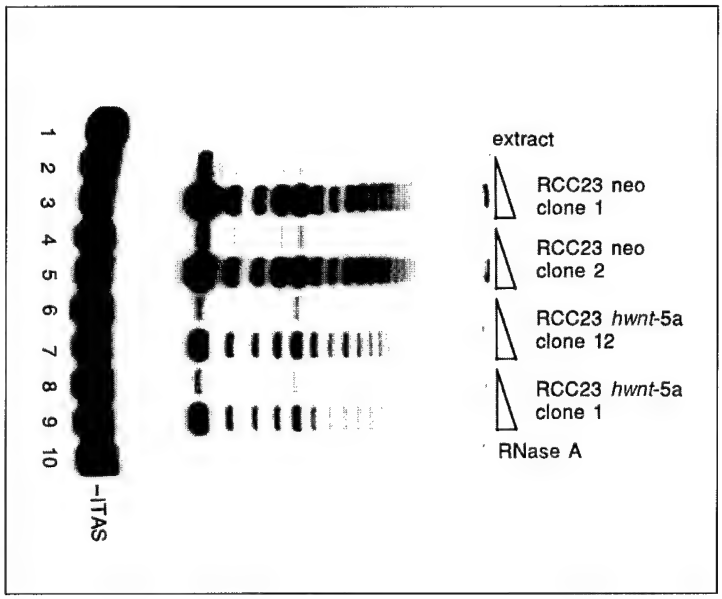

Fig. 4. Telomerase activity of RCC23/neo cells compared to RCC23/*wnt*-5a cells. Extract from RCC23/neo cells (lanes 2–5) shows a 5-fold difference in telomerase activity as measured by the TRAP assay when compared to two different clones of RCC23/*wnt*-5a cells (lanes 6–9). Controls include (lane 1) without any extract and 0.01 µg RCC23 cell extract pretreated with 200 µg/ml RNase A for 30 min at 37°C (lane 10). The triangle represents increasing amounts of extract assayed (0.01, 0.05 µg). The assays were done at least three times with similar results. To quantitate the levels of telomerase activity, the average optical density of first six TRAP bands after primer band was used as a ratio to ITAS band.

RCC23 cells to two RCC23 cell lines expressing *wnt*-5a by the primer extension TRAP assay in which telomerase synthesizes telomeric repeats onto oligonucleotide primers. Results indicate a 5-fold inhibition of telomerase activity in both RCC23/*wnt*-5a expressing clones as shown in figure 4, comparing lanes 3, 5, 7, and 9.

Discussion

Functional inactivation of tumor suppressor genes is hypothesized to be important in the multi-progressive pathways leading to human tumorigenesis [5]. The high frequency of loss and rearrangement of chromosome 3p in many different types of human cancer including renal cell carcinoma suggest that one or more tumor suppressor genes map to the short arm of chromosome 3 (3p) [1–4]. Although the precise localization of the suppressor gene(s) is unknown, 3p11-p25 has been designated for possible tumor suppressor gene localization [7–12]. We are particularly interested in searching for genes in this region, which is known to include *wnt*-5a (3p14-p21) [24, 25]. Previous studies have indicated that *wnt*-5a is a potential growth-regulating gene in mouse mammary epithelial cells. This has been more directly addressed recently using antisense *wnt*-5a DNA which was transfected into normal C57MG cells resulting in *wnt*-1-like transformation [33, Olson et al. unpubl. data]. It is of interest that human *wnt*-5a in several normal and malignant cell lines is aberrantly expressed without gene amplification or rearrangement compared to corresponding normal cell lines [34]. In addition, in breast cancer tissue, *wnt*-5a expression is up-regulated in malignant breast cancer but dramatically downregulated when compared to corresponding benign tissue [25]. It may be that the upregulation of *wnt*-5a gene expression in malignant and benign tissue relates to an attempt to restore normal growth control

by regulating cell migration. It has been reported that *wnt*-5a controls cell migration by its up-regulation in cell culture at confluence [23].

The nontumorigenic renal cell carcinoma cell line (RCC23) has been previously established from a stage III nonpapillary carcinoma [27]. Chromosome and RFLP analysis revealed an unbalanced translocation between chromosome 3p and 8q (t(3;8)(p11;q11)) resulting in the loss of the 3p11-pter region [27]. Using microcell-mediated chromosome transfer, chromosome 3 was reintroduced into the RCC23 cells, resulting in hybrids with flattened cell morphology, decreased cell saturation density, and increased population doubling time [27]. The results in this study with RCC23 cells that are transfected with *wnt*-5a are very similar to that found previously in the hybrids introduced with chromosome 3. That is, the morphologic phenotype and growth characteristics are virtually indistinguishable between RCC23 hybrids with introduced chromosome 3 and RCC23 cells transfected with *wnt*-5a. This suggests that *wnt*-5a regulates cell growth and may be a candidate tumor suppressor gene on chromosome 3p. It is not possible to directly address this using RCC23 renal cell carcinoma cells which are nontumorigenic in athymic nude mice and do not grow in anchorage-independent assays. However, recent data using a human uroepithelial cancer line with LOH for chromosome 3p13-21.2 and transfected with human *wnt*-5a suppresses tumor growth when grown in nude mice [26], supporting the hypothesis that *wnt*-5a not only regulates cell growth but also functions as a tumor suppressor gene.

Telomerase activity is thought to correlate with cell immortalization, cell transformation, and with tumorigenesis [35]. Most human tumor cells have enhanced telomerase activity when compared to normal somatic cells [36]. Differentiation of tumor cells has been found to correlate with repression of telomerase activity [37]. The present study demonstrates that transfection of human *wnt*-5a into renal cell carcinoma cells (RCC23) missing chromosome 3p clearly represses telomerase activity. This mimics the finding that RCC23 microcell hybrids introduced with chromosome 3 have repressed telomerase activity [27]. It is of interest that *wnt*-1, known to suppress the gene expression of *wnt*-5a [22] enhances telomerase activity in mouse mammary tumors [32]. One difference in our study, however, is that *wnt*-5a did not shorten telomeric length unlike that seen in the RCC23 hybrids. One explanation is that other regulating factors are involved in reestablishing cell senescence that are located on chromosome 3 which do not involve *wnt*-5a. Furthermore, it has been reported that repression of telomerase activity does not necessarily correlate with shortening of telomeric length [37, 38]. No specific regulators of telomerase activity mapped to chromosome 3p have previously been described. RCC23 hybrids introduced with chromosome 3 have repressed telomerase activity which indicates a regulatory gene(s) localized to this region. In support of this, by deletion mapping a telomerase repressor gene has been localized to chromosome 3p14-p21 [Oshimura, unpubl. data]. Our study suggests that *wnt*-5a which maps to chromosome 3p14-p21 could be a regulator of telomerase.

We have shown that *wnt*-5a when ectopically expressed in RCC23 renal cancer cells missing chromosome 3p reverts the transformed phenotype and represses telomerase activity in a manner similar to that when intact chromosome 3 is introduced. This indicates that the deregulated expression of *wnt*-5a may be important in the multi-step progression of renal cancer. These results have important implications for understanding

basic mechanisms underlying tumorigenesis and it may be that *wnt*-5a is one of the candidate tumor suppressor genes mapped to chromosome 3p which is rearranged or deleted in renal cell carcinoma, and possibly other types of human cancer.

Acknowledgments

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Reversion of Uroepithelial Cell Tumorigenesis by the Ectopic Expression of Human *wnt*-5a[1]

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Abstract

***Wnt* gene family members are thought to play an important role in cell growth and differentiation. When normal *wnt* gene expression is disrupted, there is the potential for cell transformation. We have found previously that a strong correlation exists between the loss of normal *wnt*-5a gene expression and cell transformation (Olson and Papkoff, Cell Growth & Differ., *5:* 197–206, 1994). Recently, this has been tested directly using antisense *wnt*-5a, which, when expressed in mouse mammary cells, results in cell transformation (Olson and Gibo, Antisense *wnt*-5a transforms C57MG mouse mammary epithelial cells, manuscript in preparation). We hypothesize that *wnt*-5a is a growth-regulating gene, the disruption of which could result in tumorigenesis. The multistage progression of many human cancers involves the loss of normal tumor suppressor gene(s) activity. Several tumor suppressor genes are thought to map to chromosome 3p11–p25. We have studied the ectopic expression of human *wnt*-5a (3p14–p21) in a tumorigenic uroepithelial cell line with deletion of chromosome 3p13–p21.2. This results in loss of tumorigenicity in athymic nude mice and suppresses anchorage-independent cell growth in soft agar. This suggests that human *wnt*-5a is a novel tumor suppressor gene in uroepithelial cell carcinoma and may be one of the suppressor genes deleted or rearranged on chromosome 3p.**

Introduction

Genetic alterations of chromosomes containing tumor suppressor genes are thought to be contributing to the multistage progression of malignant tumors (1). These alterations may include nonrandom chromosomal deletions or loss of heterozygosity (2). The short arm of chromosome 3 (3p) has a particularly high frequency of deletion or rearrangement in human cancers, including small cell lung carcinoma, oral squamous cell carcinoma, cervical carcinoma, breast carcinoma, renal cell carcinoma, and uroepithelial cell carcinoma (3–6). The loss of heterozygosity determined by RFLP analysis occurs consistently in renal cell carcinoma, suggesting the loss of one or more tumor suppressor genes that likely play a significant role in renal cell carcinogenesis (7). Although the precise location of the 3p tumor suppressor gene(s) is not known, cytogenetic analysis suggests that the region 3p11–p25 likely carries one or more suppressor genes (4, 8, 9). More specifically, Yamakawa *et al.* (10) mapped one suppressor gene to 3p13–p14.2 and another distal to 3p21.3 in renal cell carcinoma. Another region encompassing 3p12–p14 has been found to dramatically alter tumor growth in nude mice when a fragment containing this region was introduced into a highly malignant nonpapillary renal cell carcinoma cell line (11). In bladder cancers, a specific correlation between the loss of chromosome 3p and the development of high-grade malignancy has recently been found (12).

Human *wnt*-5a has been cloned and mapped to chromosome 3p14–p21 (13). Recently, we have found that human renal cell carcinoma cells missing chromosome 3p (RCC23; Ref. 14) and transfected with *wnt*-5a differentiate the transformed phenotype and repress telomerase activity.[5] *Wnt* genes consist of a family of locally acting growth factor-like molecules that are involved in pattern formation, morphogenesis, and cell growth and differentiation (15). *Wnt*-1, *wnt*-2, and *wnt*-3 are known to be activated by mouse mammary tumor virus proviral insertional mutagenesis in certain mouse mammary tumors (15). It is not known how some *wnt* gene family members are involved in cell transformation and tumorigenesis, but it likely involves disruption of normal spatiotemporal *wnt* gene expression. In support of this, Olson and Papkoff (16) previously found that *wnt*-5a endogenously expressed in mouse mammary epithelial cells may control normal cell growth. That is, loss of normal gene expression of *wnt*-5a in the presence of ectopically expressed *wnt*-1, *wnt*-2, or *neu* T correlates with cell transformation (16). This has recently been extended directly using antisense *wnt*-5a,

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[1] This research was supported in part by the Department of the Army Grant DAMD17-94-J-4150 (to D. J. O.).
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[5] D. J. Olson, M. Oshimura, A. P. Otte, and R. Kumar. Ectopic expression of *wnt*-5a in human renal cell carcinoma suppresses *in vitro* growth and telomerase activity, submitted for publication.

which results in the loss of normal *wnt*-5a gene expression and leads to cell transformation.[6]

We have been studying an SV40-immortalized human uroepithelial cell line (SV-HUC-1) that showed nonrandom losses of chromosome 3p in association with tumorigenic transformation to high-grade cancers (17). In support of the hypothesis that genes on chromosome 3p act as tumor suppressors, Wu *et al.* (18) showed that all somatic cell hybrids that were formed between nontumorigenic SV-HUC-1 cells and an isogeneic derivative transitional cell carcinoma cell line (MC-T16) that lost 3p on initial transformation were tumorigenically suppressed. Upon reversion, hybrids were subsequently found to have a deletion of chromosome 3p13–p21.2 (19). In the present study, we investigated whether human *wnt*-5a is a growth-regulating gene important to the progression of uroepithelial cell carcinoma, as suggested by its strategic chromosome localization. We have stably transfected human *wnt*-5a into MC-T16 cells, which results in loss of tumorigenicity in athymic nude mice and suppresses anchorage-independent cell growth in soft agar. This suggests that human *wnt*-5a is a novel tumor suppressor gene in uroepithelial cell carcinoma and may be one of the suppressor genes deleted or rearranged on chromosome 3p.

Results

Analysis of MC-T16 Cells Transfected with *wnt*-5a. Initially, transient expression assays were done on SV-HUC-1 and MC-T16 cells to determine the effect of the pRSV mammalian expression vector on cell phenotype and to determine the concentration of cDNA tolerated by both cell lines. It was found that transfection of the pRSV vector alone did not change cell phenotype for either cell line. It was also determined that a total of 10 μg of cDNA was well tolerated. For stable gene expression, uroepithelial carcinoma cells (MC-T16) were then transfected with full-length human *wnt*-5a and/or pSV2neo using liposomes (Lipofectin, Life Technologies, Inc.). Clones were stably selected in G-418, isolated, and expanded into stable cell lines. Northern analysis using a random primed *wnt*-5a cDNA ^{32}P-labeled probe demonstrated expression of the expected 3.2-kb RNA in several clonally expanded cell lines. Two clones expressing *wnt*-5a shown in Fig. 1*a* (*clone 900, Lane 1,* and *clone 6, Lane 2*) were selected for comparison to the parental MC-T16/neo-transformed cells (*Lane 3*) and SV-HUC-1/neo nontumorigenic immortal cells (*Lane 4*) resistant to G-418.

***In Vitro* Growth Assays.** The growth properties were compared between the MC-T16/neo parental cells and two clones of MC-T16/*wnt*-5a cells to determine whether *wnt*-5a had any influence on growth kinetics. As shown in Table 1 and Fig. 1*b*, the growth rate of the MC-T16/neo cells expressing *wnt*-5a was similar to MC-T16 parental cells in the logarithmic phase, unlike the growth rate of SV-HUC-1/neo cells. That is, the population doubling time of MC-T16/neo cells was 26 h, whereas that for MC-T16/*wnt*-5a-clone 6 cells was 32 h and for MC-T16/*wnt*-5a-clone 900 was 24 h, in comparison to the doubling time for SV-HUC-1/neo cells, which was 56 h. Cell saturation density at confluence correlated with growth rate. That is, MC-T16/neo parental cells had a saturation density of 8.9×10^5 at confluence, whereas MC-T16 *wnt*-5a clone 6 and MCT16/*wnt*-5a clone 900 had saturation densities of 9.4×10^5 and 8.0×10^5, respectively. This was significantly different from that observed for SV-HUC-1/neo cells, which had a saturation density of 3.2×10^5. Comparing these results with the incorporation of [^{3}H]thymidine (Table 1) suggests that the expression of *wnt*-5a in MC-T16 cells does not alter growth kinetics significantly.

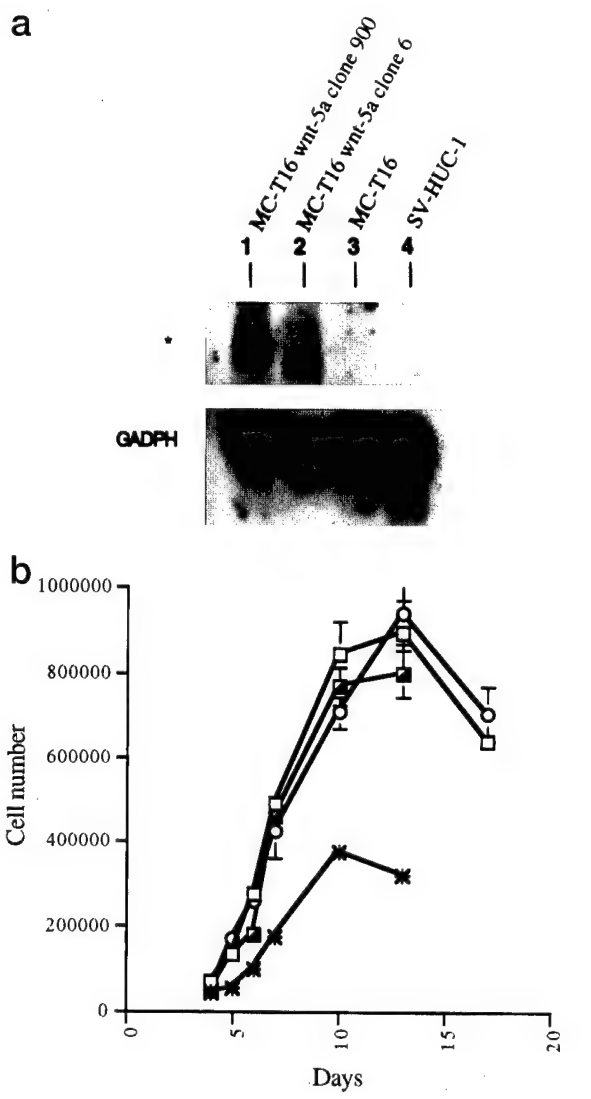

Fig. 1. Characterization of MC-T16/*wnt*-5a cells. *a*, Northern analysis of MC-T16/*wnt*-5a-clone 900 (*Lane 1*) and MC-T16/*wnt*-5a-clone 6 (*Lane 2*) stably expressing *wnt*-5a at the predicted 3200-bp size compared to MC-T16 cells (*Lane 3*) and SV-HUC-1 cells (*Lane 4*). The same blot was probed for GADPH for RNA loading and integrity. *b*, growth curves for MC-T16/*wnt*-5a-clone 6 (○) and MC-T16/*wnt*-5a-clone 900 (◪) cells were compared to those for MC-T16/neo cells (□) and parental SV-HUC-1/neo cells (*). *Data points*, mean of at least three dishes; *bars*, SE.

[6] D. J. Olson and D. M. Gibo, Antisense *wnt*-5a transforms C57MG mouse mammary epithelial cells, manuscript in preparation.

Table 1 Growth characteristics of MC-T16 cells transfected with human wnt-5a

Cells at passage 18–20 were seeded at 4×10^4 cells per 5-cm plastic dish for thymidine incorporation or in 12-well plastic dishes for growth kinetics and saturation density. The saturation density was determined on day 13. To determine growth in 0.35% soft agar, 10^4 cells were suspended and colony formation was determined at 2 weeks. The values shown are mean ± SE of at least three dishes. The experiments were repeated three times with similar results.

Cell type	Generation time (h)	Saturation density (total cells $\times 10^5$)	[^{3}H]thymidine incorporation (cpm)	Colony formation (%)
SV-HUC-1	56	3.2 ± 0.1	8,212	0
MC-T16	26	8.9 ± 0.8	15,110	9.7 ± 0.3
MC-T16 hwnt-5a-6	32	9.4 ± 0.7	11,420	0
MC-T16 hwnt-5a-900	24	8.0 ± 0.6	10,435	0.06 ± 0.03

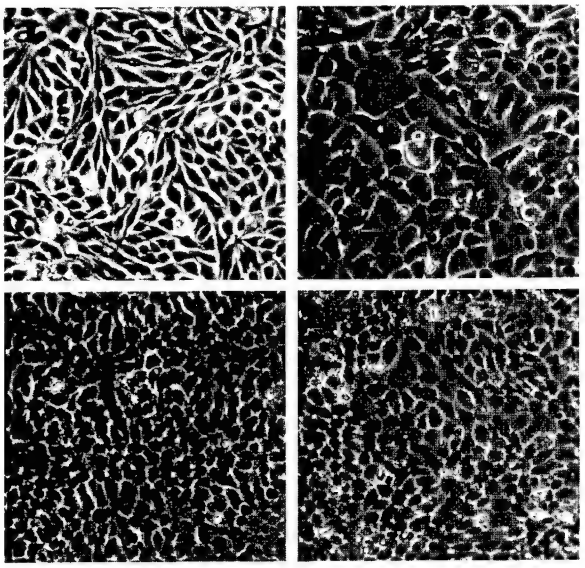

Fig. 2. Photomicrographs of confluent cells comparing MC-T16/*wnt*-5a cells to MC-T16/neo and SV-HUC-1/neo cells. MC-T16/neo cells (*a*) are refractile and elongated and grow in nests, unlike SV-HUC-1/neo cells (*b*), which are large, flat, polygonal cells. MC-T16/*wnt*-5a-clone 900 cells (*c*) and MC-T16/*wnt*-5a-clone 6 cells (*d*) no longer have the morphology of MC-T16/neo cells and appear somewhat similar to the phenotype of SV-HUC-1/neo cells.

Morphological Differences in MC-T16/*wnt*-5a Cells. SV-HUC-1/neo cells characteristically retain many of the features associated with normal epithelial cells in culture. The isolated SV-HUC-1/neo clone used for these experiments no longer grew at confluence (Fig. 2*b*), unlike SV-HUC-1/neo pooled clones, which become tightly packed at confluence.[7] MC-T16/neo carcinoma cells continue to grow at confluence, are refractive, and have a spindle cell phenotype (Fig. 2*a*). However, when two different clones of MC-T16/*wnt*-5a cells were examined [Fig. 2, *c* (MC-T16/*wnt*-5a-clone 900) and *d* (MC-T16/*wnt*-5a-clone 6)], the cells at confluence become less spindle shaped, more flattened, and pleiomorphic similar to the SV-HUC-1/neo cells. These findings suggest that MC-T16 bladder cancer cells ectopically expressing *wnt*-5a are more differentiated than the MC-T16/neo cells.

[7] Unpublished observations.

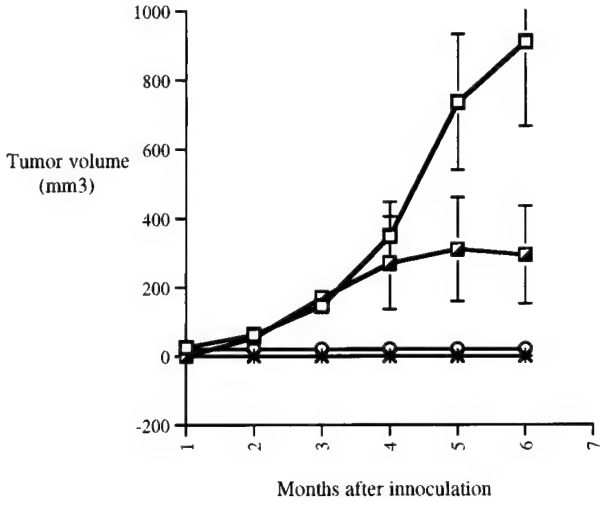

Fig. 3. Tumor volumes of MC-T16/neo cells inoculated into athymic nude mice were compared to SV-HUC-1/neo cells and MC-T16/*wnt*-5a-expressing cells. No tumor growth occurred in 10 nude mice inoculated with SV-HUC-1/neo cells (*) or in 10 nude mice inoculated with MC-T16/*wnt*-5a-clone 6 cells (○). After a lag period of 4 weeks, tumors began to grow slowly in mice inoculated with MC-T16/neo cells (□); the growth then rapidly accelerated after 3 months. This is compared to tumor development following inoculation of MC-T16/*wnt*-5a-clone 900 cells (◪), which had a lag time of 6 weeks before the onset of tumor formation. These tumors also grew slowly and at 3 months stopped growing. *Data points*, mean tumor volume; *bars*, SE.

Anchorage Dependence Assay. Parental MC-T16/neo-transformed cells grow in 0.35% agar with a cloning efficiency of 9.7%, compared to no growth in corresponding parental G-418 selected SV-HUC-1/neo cells (Table 1). The expression of *wnt*-5a in MC-T16 cells reestablishes contact-dependent growth under these conditions in the two different *wnt*-5a-expressing clones. The experiments were repeated three times with similar results.

Tumorigenesis in Athymic Nude Mice. s.c. inoculation of MC-T16/neo uroepithelial cells resulted in tumor formation after 4 weeks in 9 of 10 animals. Spontaneous tumor regression occurred in four mice, which has been described previously for tumors derived from this cell line (18), and one 1 × 1-cm tumor was removed for analysis before the end of the experiment. The remaining tumors grew slowly until the 3rd month, when tumor growth rapidly accelerated (Fig. 3). These tumors were removed when they were greater than 1.5 × 1.5 cm or when became necrotic. No tumors grew in 10 mice inoculated with SV-HUC-1/neo cells or in 10 mice inoculated with MC-T16/*wnt*-5a-clone 6 cells. However, 5 of 5 mice inoculated with MC-T16/*wnt*-5a-clone 900 cells grew tumor after a lag time of 6 weeks. Tumor regression occurred in one animal and one 0.6 × 0.8-cm necrotic tumor was removed from another animal. At three months, these tumors grew to a maximum size that never enlarged to greater than 1 × 1 cm over the following 3 months (Table 2). The tumors

Table 2 Tumorigenicity of human uroepithelial cells in athymic nude mice

Cells at passage 18–20 were grown, and 2 × 10^6 cells in 0.1 ml PBS were injected s.c into the right dorsal quadrant of 4–6-week-old female athymic nude mice.

Cell line	Tumor formation (no. of mice)	Tumor regression (no. of mice)	Tumor size (no. >1 × 1 cm)
SV-HUC-1	0/10	0/0	0/0
MC-T16	9/10	4/9	4/5
MC-T16 hwnt-5a-6	0/10	0/0	0/0
MC-T16 hwnt-5a-900	5/5	1/5	0/4

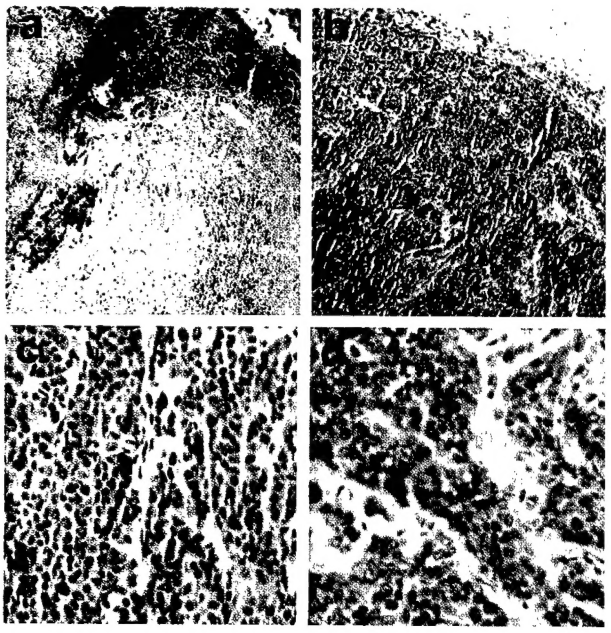

Fig. 4. Photomicrographs of tumors removed from nude mice to compare histopathology. *a*, tumor formed as a result of inoculation of MC-T16/*wnt*-5a-clone 900 cells has extensive cell death compared to a MC-T16/neo-derived tumor of similar size (*b*), which has no cell death. *c*, higher magnification of the tumor derived from MC-T16/*wnt*-5a-clone 900 cells demonstrates little stroma formation compared to a MC-T16/neo cell-derived tumor with extensive stromal development (*d*).

that were examined from mice inoculated with MC-T16/neo cells were attached to underlying tissue and were vascular, whereas the tumors that grew in mice inoculated with MC-T16/*wnt*-5a-clone 900 cells were found to be unattached to surrounding tissues and remarkably avascular. Tumors from both groups examined histologically confirmed the gross findings. Furthermore, the tumors expressing *wnt*-5a were 80–90% necrotic centrally (Fig. 4*a*), compared to MC-T16/neo cell-derived tumors, which were 10–20% necrotic; this contrast is striking even considering that the latter tumors grew to a much larger size (Fig. 4*b*). At higher magnification, little stroma was apparent in MC-T16/*wnt*-5a-clone 900-derived (Fig. 4*c*) tumors compared to MC-T16/neo tumors (Fig. 4*d*).

Telomerase Assay. Reports have shown that the process of transformation/neoplasia is associated with the activation of telomerase, a ribonucleoprotein enzyme complex that adds telomeric repeats (hexanucleotide 5′-TTAGGG-3′) to the ends of replicating chromosomes, or telomeres, and that another member of the *Wnt* family (20) has been shown to regulate telomerase. We compared telomerase activity between two different G-418-resistant clones of parental MC-T16/neo cells and the two clones of MC-T16/*wnt*-5a-expressing cells by the primer extension TRAP[8] assay, in which telomerase synthesizes telomeric repeats onto oligonucleotide primers (21). Results indicate no change in telomerase activity in either MC-T16/*wnt*-5a-expressing cell line (Fig. 5).

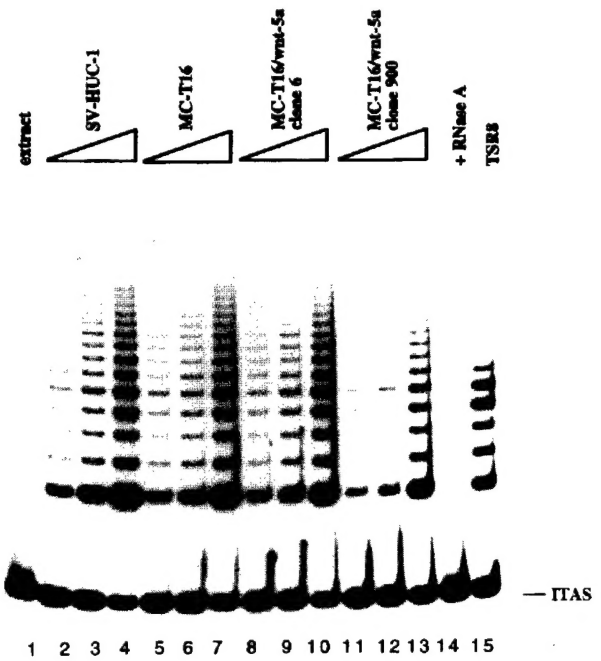

Fig. 5. Telomerase activity of MC-T16/neo cells compared to MC-T16/*wnt*-5a cells and SV-HUC-1/neo parental cells. Extract from SV-HUC-1/neo cells (*Lanes 2–4*) shows no difference in telomerase activity as measured by the TRAP assay when compared to MC-T16/neo cells (*Lanes 5–7*), MC-T16/*wnt*-5a-clone 6 (*Lanes 8–10*), or MC-T16/*wnt*-5a-clone 900 (*Lanes 11–13*). Controls were: *Lane 1*, tube without any extract; *Lane 14*, 0.1 μg of SV-HUC-1 cell extract pretreated with 200 μg/ml RNase A for 30 min at 37°C; and *Lane 15*, positive assay control with 2 μl of TSR8 template. *Triangles*, increasing amounts of extract assayed (0.01, 0.05, and 0.1 μg). The assays were done at least three times with similar results.

Discussion

In this study, we examined MC-T16 cells with allelic deletions for chromosome 3p13–21.2 that underwent transfection with *wnt*-5a. The transgene has caused significant alteration in the transformed phenotype. That is, MC-T16/*wnt*-5a-expressing cells at confluence become polygonal and flatten similarly to the parental SV-HUC-1/neo cells. In addition, MC-T16/*wnt*-5a cells no longer grow in anchorage-independent conditions, and when these cells are inoculated into

[8] The abbreviations used are: TRAP, telomere repeat amplification protocol; FBS, fetal bovine serum; ITAS, internal TRAP assay standard.

athymic nude mice, tumor growth is suppressed, indicating the potential for *wnt*-5a as a tumor suppressor gene.

Functional inactivation of tumor suppressor genes is hypothesized to be important in the multiprogressive pathways leading to human tumorigensis (1). The high frequency of loss and rearrangement of chromosome 3p in many different types of human cancer, including uroepithelial cell carcinoma, suggests that one or more tumor suppressor genes map to the short arm of chromosome 3 (3p). Although the precise mapping of the suppressor gene(s) is unknown, region 3p12–p14.2 has been designated for possible tumor suppressor gene localization. We have been particularly interested in searching for candidate tumor suppressor genes mapped to this region, including *wnt*-5a (3p14–p21; Ref. 13).

Previous studies have indicated that *wnt*-5a is a potential growth-regulating gene in C57MG mouse mammary epithelial cells. In *wnt*-1-transformed C57MG cells, the gene expression of *wnt*-5a is reduced 2–3-fold, and in *neu* T-transformed C57MG cells, the expression is reduced 20–25-fold (16). This has been more directly addressed recently using antisense *wnt*-5a DNA, which was transfected into normal C57MG cells, resulting in *wnt*-1-like transformation. That is, the cells continue to grow at confluence and morphologically become similar to the *wnt*-1-transformed cell phenotype.[5] Loss of normal *wnt*-5a gene expression in mouse cells correlates with increased cell proliferation and increased cell saturation density.[9] It is also of interest that human *wnt*-5a in several malignant cell lines is minimally expressed or aberrantly expressed compared to corresponding normal cell lines (22, 23). Although *wnt*-5a gene expression in breast cancer tissue is somewhat elevated, its expression is markedly reduced when compared to corresponding benign tissue (22). The fact that *wnt*-5a expression is elevated in benign tumors, including colon adenomas, suggests that *wnt*-5a may be functionally related to early progression of tumorigenesis (24). It is also possible that *wnt*-5a regulates normal cell growth and that its expression will transiently increase in transformed cells, which reflects an attempt to reestablish normalcy. In support of this notion, it has been recently shown that *wnt*-5a gene expression increases at confluence in human breast epithelial cells. It is speculated that this increase in *wnt*-5a expression is an attempt to inhibit cell migration at confluence to regulate cell growth (25). Although the function and mechanism(s) of action of *wnt*-5a are not known, it appears that disruption of normal *wnt*-5a gene expression correlates with abnormal cell growth, which may progress to tumorigenesis.

In this study, the ectopic expression of *wnt*-5a did not change the growth kinetic parameters compared to the MC-T16 carcinoma cell line. That is, cell saturation density at confluence and population doubling times during log phase did not change significantly. However, anchorage-independent growth was reverted in two MC-T16 clones expressing *wnt*-5a. This suggests that *wnt*-5a signaling involves pathways that may affect cell adhesion molecules and not necessarily alter cell proliferation resulting from SV40-mediated immortalization (24). In support of this, it has been reported that ectopic *wnt*-5a expression in developing *Xenopus* embryos alters cell migration during gastrulation (26, 27). These effects may reflect alterations in cell adhesion due to *wnt*-5a because *wnt*-5a decreases Ca^{2+}-dependent cell adhesion in a manner similar to a mutant form of N-cadherin that inhibits cadherin-mediated cell adhesion (28, 29). These changes apparently can take place without blocking cell fate (26, 27). This is interesting considering that although the onset of tumor growth of MC-T16-*wnt*-5a-clone 900 cells in nude mice was somewhat delayed and although *wnt*-5a repressed tumorigenesis, the cell phenotype was apparently unaffected. That is, MC-T16 tumor histopathology of uroepithelial cells was not altered in the presence of *wnt*-5a. Although we do not know how *wnt*-5a is able to suppress tumorigenicity, it is of interest that the MC-T16/*wnt*-5a cells that did form tumors, formed tumors that were relatively avascular with extensive necrosis compared to the parental MC-T16/neo-derived tumors. This raises the possibility that *wnt*-5a signaling involves pathways important for neo-angiogenesis. Interestingly, the tumors formed from MC-T16/*wnt*-5a cells also were remarkably devoid of stroma. The relationship between stroma, cell adhesion molecules, and angiogenesis is well documented (30). We are currently exploring the possibility that *wnt*-5a compromises angiogenesis by altering cell adhesion, which would offer an explanation of why the other MC-T16/*wnt*-5a-expressing clone did not grow at all in athymic nude mice.

Telomerase activity is believed to correlate with cell immortalization, cell transformation, and tumorigenesis. Telomerase is a ribonucleoprotein complex that adds telomeric repeats to chromosomal ends. This is thought to protect the ends against nucleases and ligases, thus maintaining stability, and to counter shortening of telomere length as a result of DNA replication (31). Most human tumor cells have enhanced telomerase activity when compared to normal somatic cells (32). It has recently been reported that telomerase is activated in *wnt*-1-mediated mouse mammary tumors (20). This is interesting considering that the endogenous expression of *wnt*-5a decreases 2–3-fold in *wnt*-1-transformed C57MG mouse mammary epithelial cells. Because differentiation of tumor cells has been found to correlate with repression of telomerase activity (21), we wanted to determine the effect of *wnt*-5a on telomerase activity. In addition, we have recently found that when *wnt*-5a is transfected into nontumorigenic renal cell carcinoma cells (RCC23), telomerase activity is repressed.[5] The present study, however, demonstrates that transfection of human *wnt*-5a into uroepithelial carcinoma cells (MC-T16) missing chromosome 3p13–21.2 does not repress telomerase activity. One explanation is that other regulating factors are involved and are unique to each type of cell line. A more plausible idea is that the parental cell line (SV-HUC-1), unlike RCC23 cells, has been immortalized by SV40, which has previously been shown to activate telomerase and stabilize telomere shortening (33). There is also evidence for up-regulation of telomerase RNA, which is dependent on cell proliferation (20). Furthermore, telomerase activity in tumor cells increases during S phase, and less activity is detected during G_0/G_1 (34, 35). It would be of

[9] Unpublished data.

interest to determine whether SV40 alters cell-cycle-dependent telomerase activity.

We have shown that *wnt*-5a, when ectopically expressed in MC-T16 uroepithelial cancer cells, causes the transformed phenotype to revert. Anchorage-independent growth is lost and tumor growth in athymic nude mice is suppressed or altered. We suggest that *wnt*-5a is capable of suppressing tumorigenesis in human bladder cancer by compromising angiogenesis, resulting in extensive cell death and limiting tumor growth. Whether these changes relate to pathways involving cell adhesion or compromise angiogenesis directly is currently being investigated. These results have important implications for understanding basic mechanisms underlying tumorigenesis and indicate that the deregulated expression of *wnt*-5a may be important in the multistep progression of cancer. It may be that *wnt*-5a is one of the candidate tumor suppressor genes mapped to chromosome 3p that is rearranged or deleted in many different types of human cancer.

Materials and Methods

Transfection and Cell Characterization. Full-length human *wnt*-5a cDNA (clone T11; Ref. 13) was the kind gift of Dr. Renato Iozzo (Jefferson Medical College, Philadelphia, PA). The cDNA was subcloned into pRSV (Dr. Jackie Papkoff, Sugen, Redwood City, CA) and orientation was determined by restriction analysis. Initially, transient expression studies were done by transfecting pRSV/*wnt*-5a or pRSV alone into previously characterized SV-HUC-1 parental uroepithelial cells and MC-T16 uroepithelial carcinoma cells (Ref. 19; a gift of Dr. Catherine Reznikoff, University of Wisconsin, Madison, WI). This was done to determine the effect of the pRSV expression vector on cell phenotype and to determine the concentration of cDNA tolerated by these cell lines. Cells were cultured in F12 medium supplemented with 1% FBS (Life Technologies, Inc.) and 1% penicillin/streptomycin. The medium was also supplemented with essential amino acids, ferritin, dexamethasone, insulin, and glutamine as described previously. Cells were grown to 80% confluence before each passage. For stable gene transfection, passage 20 cells were grown to 50% confluence, and the medium was exchanged for low-serum medium (Optimem, Life Technologies, Inc.). Using 90 μl of liposomes (Lipofectin, Life Technologies, Inc.) mixed with 10 μg of pRSV*wnt*-5a and/or pSV2neo in a total volume of 150 μl, the cells were transfected overnight at 37°C in 5% CO_2. The medium was replaced, and the cells were grown overnight in medium with 1% FBS without the addition of Geneticin (G-418, Life Technologies, Inc.). The cells were then selected in medium supplemented with 800 μg/ml G-418. Individual clones were isolated and resistant colonies expanded into cell lines and maintained in medium supplemented with 250 μg/ml G-418 for eventual RNA extraction to determine gene expression of *wnt*-5a.

To determine the growth rates, cell saturation density, population doubling time, and morphological phenotype of SV-HUC-1/neo cells, MC-T16/neo cells and MC-T16/*wnt*-5a cells, cells were plated in 12-well dishes at a density of 4×10^4 cells per well. The cells were counted every 2 days for 17 days, and the growth rate and population doubling time were determined from the logarithmic part of the growth curve. The saturation density was determined from the cell number after the cells reached confluence. Morphology was determined by growing cells to confluence and photographing them.

RNA Isolation and Analysis. Total cellular RNA was isolated from dishes of confluent cells (36). Twenty μg of RNA were analyzed on a 1.0% agarose formaldehyde gel followed by transfer to Hybond-N (Amersham Corp.) membrane. The membranes were UV cross-linked (Stratagene) and prehybridized at 42°C for 3–6 h and then hybridized at 42°C overnight with full-length cDNA *wnt*-5a probes labeled by random priming with [^{32}P]dCTP using $1–2 \times 10^6$ cpm/ml. The prehybridization and hybridization solutions consisted of 50% formamide, 4× saline-sodium phosphate-EDTA, 0.2 mg/ml sheared and boiled salmon sperm DNA, 2.5× Denhardt's solution, and 1% SDS. Membranes were washed at room temperature twice in 2× SSC, 1% SDS, followed by several washes in 0.1× SSC, 0.1% SDS at 55°C. The membranes were then mounted on 8 × 10 film (Kodak) with an intensifying screen and placed at −80°C up to 5 days.

[^{3}H]Thymidine Incorporation. Cell lines were plated into 5-cm dishes in standard growth medium and grown to confluence for 14 days; the medium was changed every 2 days. In triplicate, 4 μCi of [^{3}H]thymidine (methyl-^{3}H, 60–90 Ci/mmol, aqueous; ICN Radiochemicals) were added to each dish, and the dish was incubated for 2 h at 37°C. The cells were washed twice with PBS followed by addition of 2 ml ice cold 10% trichloroacetic acid and incubated for 30 min on ice. Cells were then washed with 10% trichloroacetic acid followed by addition of 2 ml of 0.1 N NaOH. The dishes were incubated at 37°C for 30 min and then neutralized with 0.2 ml of 1 N HCl. The dishes were carefully scraped, and the extract was added to 10 ml of scintillation fluid for counting. A fourth dish of cells was grown in parallel to determine total protein and the counts normalized to total protein. The results reflect the summary of three separate experiments.

Soft Agar Assay. In triplicate, 10^4 cells were plated in 0.35% agar (Noble) suspension using standard medium over a previously poured 0.5% agar base in 12-well dishes. A G-418-selected SV-HUC-1/neo (17) clone that does not grow in soft agar was used as a negative control. G-418-resistant MC-T16/neo cells were used as a positive control. MC-T16/*wnt*-5a-clone 900 and MC-T16/*wnt*-5a-clone 6 were plated for comparison. Colony formation was determined daily for 2 weeks. Medium was added to the wells as needed. Three separate soft agar assays were done as above, and the results were pooled.

Athymic Nude Mice. The tumorigenic potentials of G-418-selected positive control MC-T16/neo cells, negative control SV-HUC-1/neo cells, MC-T16/*wnt*-5a-clone 900, and MC-T16/*wnt*-5a-clone 6 were tested by inoculation into 4–6 week old female athymic nude mice (Charles River). Mice were housed in sterile bubbles in a temperature- and humidity-controlled room. Inoculations of 2×10^6 cells/site in a total volume of 0.1 ml were made s.c. in the right dorsal quadrant as described previously (37). The animals were examined weekly. Tumors were removed at 6 months, when necrotic, or when 1.5–2 cm in diameter. Representative sections of tumors were fixed in formalin for histological preparation and stained with H&E. Fixed and stained tumors were examined blindly by three pathologists. Representative pieces of some tumors were used to initiate tumor cell lines using an explant technique. Tumor tissue was cut into 1-mm^2 explant fragments after being washed in PBS; these fragments were then plated onto tissue culture grade dishes in F-12 1% FBS-supplemented medium without G-418. After 1 week, the explants were grown in the presence of 250 μg/ml G-418, and the cells were expanded for later analysis.

Telomerase Assay. Two different MC-T16/neo cell lines were compared for telomerase activity to two different MC-T16/*wnt*-5a cell lines. Subconfluent cultures were used to prepare the detergent 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate extracts (38). Telomerase enzyme activity was measured by using a PCR-based TRAP kit from Oncor, Inc., per the manufacturer's instructions. Each reaction product was amplified in the presence of an ITAS (36 bp). The TRAP reaction products were separated by 10% PAGE, dried, and autoradiographed. The basal levels of telomerase activity (ladder formation) were measured by serial dilution of the protein extracts, and an appropriate range of protein concentration selected that produced a linear response as described (23). Each set of TRAP assays included control reaction tubes without any extract, extracts treated with RNase A (200 μg/ml), and positive assay control TSR8 template (2 μl/tube). To quantitate the levels of telomerase activity, the average absorbance of the first six TRAP bands after the primer band was calculated as a ratio to the ITAS band.

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